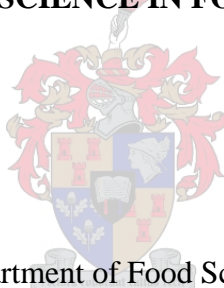


# EFFECT OF ENDOXYLANASES, ENDOGLUCANASES AND THEIR COMBINATION ON WHEAT FLOUR BREAD QUALITY

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Thesis presented in partial fulfilment of the requirements for the degree of

**MASTER OF SCIENCE IN FOOD SCIENCE**



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## **Declaration**

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Date: 25<sup>th</sup> February 2009

## Abstract

Endoxylanases are known to improve dough stability, oven spring, loaf volume, crumb structure and shelf life. The use of endoglucanases (cellulases) usually results in increased bread loaf volume, bread score and reduced crumb firmness. Even though bakeries use ‘pure’ enzymes in their formulations, they are supplied with an enzyme mixture which can contain up to five different enzymes. These mixtures often also include an emulsifier and ascorbic acid. To compare the ability of endoxylanase and endoglucanase to improve bread quality characteristics, a commercial endoxylanase (from *Aspergillus niger*) and endoglucanase (from *Trichoderma reesei*) were evaluated together with a pure endoxylanase and endoglucanase (both from *Trichoderma* sp). Baking trials were conducted on small (100 g) as well as commercial (700 g) scale. Quality characteristics evaluated included dough quality, bread weight, bread height, bread volume, softness of crumb, bread slice characteristics and overall crumb texture. All the results were compared to a control. From the results of the small-scale baking trials both the pure and commercial endoxylanases significantly ( $P<0.05$ ) improved bread height and softness of crumb, with the pure endoxylanase also increasing slice brightness. Both the pure and commercial endoglucanases significantly ( $P<0.05$ ) increased softness of the crumb and slice brightness. When the enzymes were evaluated in combination, only an increase in bread height was observed for some of the combinations.

From the results of the baking trials conducted on commercial scale, the loaf height was significantly ( $P<0.05$ ) increased by the pure endoxylanase and the pure endoglucanase, while the bread volume was significantly ( $P<0.05$ ) increased by all the enzymes being tested. Enzyme combinations resulted only in a significant ( $P<0.05$ ) increase in bread volume. The texture of the bread crumb was significantly ( $P<0.05$ ) influenced by the commercial endoxylanase, the pure endoxylanase, the pure endoglucanase as well as two of the enzyme combinations, resulting in a more open and coarse crumb texture. Slice brightness was significantly ( $P<0.05$ ) decreased by the commercial endoxylanase, the pure endoxylanase, the pure endoglucanase as well as the two enzyme combinations. Both endoxylanases and endoglucanases can therefore be used to improve bread quality characteristics such as bread height and/or volume, slice brightness and softness of crumb. However, using pure enzymes specific characteristics can be targeted. This would become more feasible if pure or single component enzymes become more readily available and cost effective to use.

Apart from testing the effect of the enzymes on bread quality characteristics using small-scale baking trials, it was shown in this study that testing of enzymes could also be efficiently conducted on commercial scale. In the latter the enzymes were being tested using commercial white bread

flour as well as a leaner formulation. The leaner formulation allowed for the effect of the enzymes to be observed more prominently. The benefit of the evaluation on commercial scale was that the effect of the enzymes was tested in a process similar to that used in industry.

## Uittreksel

Dit is bekend dat endoxilanasen deegstabiliteit, broodvolume, broodkrummeltekstuur en rakleef tyd verbeter. Die gebruik van endoglukanases (sellulasies) lei gewoonlik tot 'n verhoging in broodvolume, algehele broodkwaliteit en 'n verlaging in broodkrummelfermheid. Ten spyte daarvan dat bakkerie 'suiwer' ensieme in hul formulasies gebruik, ontvang hulle 'n ensiem mengsel wat tot vyf verskillende ensieme kan bevat. Gewoonlik bevat hierdie mengsels ook 'n emulsifiseerder en askorbiensuur. Om die vermoë van endoxilanasen en endoglukanase om kwaliteitseienskappe van brood te verbeter te vergelyk, is 'n kommersiële endoxilanasen (vanaf *Aspergillus niger*) en endoglukanase (vanaf *Trichoderma reesei*), sowel as 'n suiwer endoxilanasen en endoglukanase (beide vanaf *Trichoderma sp.*) geëvalueer. Baktoetse is uitgevoer op kleinskaal (100 g) sowel as kommersiële (700 g) skaal. Kwaliteitseienskappe geëvalueer sluit in deegkwaliteit, broodmassa, hoogte sowel as volume van die brood, sagtheid van die broodkrummel, eienskappe van 'n enkel sny brood en broodkrummeltekstuur. Alle resultate is met 'n kontrole vergelyk. Die resultate van die kleinskaal baktoetse wys daarop dat die suiwer en kommersiële endoxilanasen die brood hoogte en sagtheid van die broodkrummel betekenisvol ( $P < 0.05$ ) verhoog het. Suiwer endoxilanasen het ook die helderheid van die brood sny verhoog. Beide die suiwer en kommersiële endoglukanase het die sagtheid van die broodkrummel sowel as die helderheid van die sny brood betekenisvol ( $P < 0.05$ ) verhoog.

Toe die ensieme in kombinasie geëvalueer is, is slegs 'n verhoging in broodhoogte vir sommige van die kombinasies gesien. Die resultate van die baktoetse wat op kommersiële skaal uitgevoer is, wys daarop dat die broodhoogte betekenisvol ( $P < 0.05$ ) deur die suiwer endoxilanasen en endoglukanase verhoog is. Die broodvolume is daarenteen betekenisvol ( $P < 0.05$ ) verhoog deur al die verskillende geëvalueerde ensieme. Die verskillende ensiemkombinasies geëvalueer het slegs tot 'n betekenisvolle ( $P < 0.05$ ) verhoging in broodvolume gelei. Die tekstuur van die broodkrummel is betekenisvol ( $P < 0.05$ ) beïnvloed deur die kommersiële endoxilanasen, die suiwer endoxilanasen en endoglukanase sowel as twee ensiemkombinasies. Dit het gelei tot 'n meer oop en growwe broodkrummeltekstuur. Die helderheid van die sny brood is betekenisvol ( $P < 0.05$ ) verlaag deur die kommersiële endoxilanasen, die suiwer endoxilanasen, suiwer endoglukanase en twee ensiem kombinasies. Beide die endoxilanasen en endoglukanase kan gebruik word om die kwaliteitseienskappe van brood te verbeter in terme van broodhoogte en/of broodvolume, helderheid van die sny brood sowel as broodkrummelsagtheid. Deur suiwer ensieme te gebruik kan spesifieke eienskappe geteiken word. Die gebruik van suiwer (of enkel) ensieme sal prakties uitvoerbaar word sodra hierdie ensieme meer algemeen beskikbaar en bekostigbaar is.

Die effek van ensieme op die kwaliteitseienskappe van brood kan deur middel van kleinskaal baktoetse bepaal word, maar dit is ook in hierdie studie bewys dat ensieme effektief op kommersiële skaal getoets kan word. In laasgenoemde is die ensieme getoets deur kommersiële witbroodmeel te gebruik sowel as 'n aangepaste formulasie. Hierdie formulasie het dit moontlik gemaak om die effek van die ensieme duideliker te sien. Die voordeel van evaluasie op kommersiële skaal is dat die effek van die ensieme getoets is in 'n proses soortgelyk aan dié tans gebruik in die industrie.

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## Abbreviations

|               |   |
|---------------|---|
| AACC          | American Association of Cereal Chemists                     |
| Anon          | Anonymous   |
| ANOVA         | Analysis of variance  |
| AX            | Arabinoxylans   |
| C             | Commercial  |
| <i>ca.</i>    | <i>circa</i> (about)  |
| CBP           | Chorleywood Bread Process                                   |
| cm            | Centimeter  |
| CMC           | Chemical cellulose substrate                                |
| CP            | Calcium propionate  |
| CSL           | Calcium stearoyl lactylates                                 |
| DATEM         | Diacetyl tartaric acid esters of mono- and diglycerides     |
| DSC           | Differential scanning calorimetry                           |
| EC            | Enzyme Commission   |
| EDTA          | Ethylenediaminetetraacetic acid                             |
| e.g.          | <i>exempli gratia</i> (for example)                         |
| <i>et al.</i> | <i>et alibi</i> (and elsewhere)                             |
| EU            | European Union  |
| FDA           | Food and Drug Administration                                |
| Fig.          | Figure  |
| g             | Gram  |
| GH            | Glycoside hydrolase   |
| Glu           | Endoglucanase   |
| GMS           | Glyceryl monostearate                                       |
| GRAS          | Generally Recognised as Safe                                |
| h             | Hour  |
| HMW           | High molecular weight                                       |
| ICC           | International Association for Cereal Science and Technology |
| i.e.          | <i>id est</i> (that is)                                     |
| IUB           | International Union of Biochemistry                         |
| JECFA         | Joint Expert Committee for Food Additives                   |
| J             | Joules  |
| L             | Litre   |



|                     |   |
|---------------------|---|
| LSD                 | Least significance difference                   |
| M                   | Molar   |
| mb                  | Millibar  |
| m.b.                | Moisture basis                                  |
| mg.kg <sup>-1</sup> | Milligram per kilogram                          |
| min                 | Minutes   |
| MTI                 | Mixing tolerance index                          |
| mL                  | Millilitre                                      |
| mM                  | Millimolar                                      |
| mm                  | Millimeter                                      |
| NIR                 | Near infrared                                   |
| nm                  | Nanometer                                       |
| P                   | Pure  |
| <i>p</i> NPC        | <i>p</i> -nitrophenyl β-D-cellobioside          |
| ppm                 | Parts per million                               |
| RH                  | Relative humidity                               |
| rpm                 | Revolutions per minute                          |
| sec                 | Seconds   |
| sd                  | Standard deviation                              |
| SSL                 | Sodium stearyl lactylates                       |
| TAXI                | <i>Triticum aestivum</i> endoxylanase inhibitor |
| TL-XI               | Thaumatococcus-like xylanase inhibitor          |
| μmol                | Micromol  |
| UK                  | United Kingdom                                  |
| USA                 | United States of America                        |
| WE-AX               | Water extractable arabinoxylans                 |
| WU-AX               | Water unextractable arabinoxylans               |
| XIP                 | Endoxylanase inhibiting protein                 |
| Xyl                 | Endoxylanase                                    |

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.



# **CHAPTER 1**

## **Introduction**

## Introduction

Enzymes are used in a wide range of biotechnological processes including the baking of bread, which is one of the oldest food applications (Goesaert *et al.*, 2005). Nowadays the quality of bread is influenced by the addition of bleaching agents, oxidising agents, reducing agents, chemical preservatives and emulsifiers. Enzymes are seen as natural alternatives to additives as they are native to wheat (Linko *et al.*, 1997). The use of enzymes in baking has been studied since the 1950s, but the cost and unavailability of enzymes in large quantities hampered their widespread application.

Wheat flour contains various enzymes such as  $\alpha$ - and  $\beta$ -amylases, proteases, lipases, phosphatases, oxidases and endoxylanases (Kent & Evers, 1994). These enzymes can be constituents of wheat (indigenous), can be produced by microorganisms present in the wheat (endogenous) or can be added to the flour (exogenous) (Shalstrom & Brathen, 1997). Currently, through the process of genetic engineering, enzyme mixtures can be developed and used to replace chemical additives or give new functionalities to products (Hille, 2005). Commercial enzymes consist primarily of the same types of enzymes as those that are endogenously present in wheat (Poutanen, 1997). These commercial enzyme preparations do not exclusively contain only the specific enzyme indicated on the label, but also other enzymes that happened to be produced by the same source material/organism (Law, 2002).

Starch and protein degrading enzymes (amylases and proteases) have long been used in the baking industry (Poutanen, 1997). It was observed the loaf volume and the crumb qualities of the bread improved when commercial amylase preparations were added to the dough. This effect was due to the presence of endoxylanase contamination in the preparations (Linko *et al.*, 1997). The observation led to a shift in focus from enzymes which act on starch and proteins to enzymes that hydrolyse lignocellulose or non-starch polysaccharides (NSP).

Flour consists of starch, protein (mainly gluten), NSP, lipids, minerals and enzymes (Courtin & Delcour, 2002). Non-starch polysaccharides originate from cell wall material and include arabinoxylans (AX), arabinogalactans, cellulose,  $\beta$ -glucans, glucomannans, lignins and pectic substances (Hille & Schooneveld-Bergmans, 2004). It was reported the enzyme hydrolysis of NSP leads to the improvement of the rheological properties of dough, bread specific volume and crumb firmness (Martinez-Anaya & Jimenez, 1997). The level of hemicellulase activity in wheat flour is usually too low to deliver an optimum effect in bread making (Hille, 2005).

The most important type of hemicellulase used in bread making is therefore microbial derived endoxylanases (EC 3.2.1.8) which are able to hydrolyse the xylan backbone of AX internally (Goesaert *et al.*, 2005). At optimum levels, endoxylanases can improve dough machinability, dough

stability, oven spring, loaf volume, crumb structure and shelf life (Hamer, 1995; Poutanen, 1997). Marked increases in both oven rise and final loaf volume were described when selected endoxylanases were added to a bread formulation. These findings were further accompanied by a fine, soft crumb and increased shelf life (Popper, 1997). Work carried out by Hille and Schooneveld-Bergmans (2004) showed endoxylanases of both fungal and bacterial origin can improve bread quality in terms of loaf size and shape as well as crumb texture and softness.

Hille (2005) showed cellulases and endoxylanases work synergistically in improving bread quality characteristics such as loaf volume and crumb softness. Cellobiohydrolases, endoglucanases and  $\beta$ -glucosidases work together to break down the cellulose polymer, leaving the endoxylanases to hydrolyse the AX which is partly intertwined with the cellulose. The use of commercial cellulases as an additive in different bread making processes, resulted in increased bread loaf volumes, bread scores and reduced crumb firmness (Harada *et al.*, 2000; 2005).

When evaluating enzymes, a specific wheat cultivar, with good bread baking qualities, is usually selected and used for baking trials. Wheat flour used for bread making in South Africa is almost always a blend of different wheat cultivars. It would therefore be appropriate to use commercial white bread flour, instead of a single cultivar, when evaluating baking enzymes. In addition small-scale baking trials are typically conducted to evaluate the effect of enzymes on bread baking quality (Harada *et al.*, 2000; Harada *et al.*, 2005; Jiang *et al.*, 2005; Caballero *et al.*, 2006; Collins *et al.*, 2006; Shah *et al.*, 2006; Dornez, 2007). When conducting small-scale baking trials, a straight dough method is generally used. The formulation, processing conditions as well as equipment used in conducting a straight dough bread making method is completely different to that of commercial baking processes. Evaluating the effect of enzymes on a commercial scale, using a bread making formulation which is similar to that used during commercial baking, would thus also be advantageous. It is therefore necessary to establish if the effects of the enzymes on bread baking quality is still evident when commercial baking processes and conditions are used. Until now enzymes were usually evaluated using either pure or commercial enzymes. To be able to compare the ability of enzymes efficiently, both pure and commercial enzymes should be evaluated simultaneously during small-scale as well as commercial baking trials.

The objectives of this study were therefore to:

- evaluate the effect of pure endoxylanase (*Trichoderma reesei* Xyn2, heterologously expressed in *Aspergillus niger* D15) and pure endoglucanase (*T. reesei* egI, heterologously expressed in *A. niger* D15) as well as commercial endoxylanase (derived from *Aspergillus niger*) and commercial endoglucanase (derived from *Trichoderma reesei*) on dough and

bread quality characteristics by means of small (100 g) as well as commercial scale (700 g) baking trials; and

- evaluate the synergistic effect of the enzymes by combining the respective pure and commercial endoxylanases and endoglucanases on dough and bread quality characteristics by means of small (100 g) as well as commercial scale (700 g) baking trials.

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## **CHAPTER 2**

### **Literature**

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## Literature review

### 1. Introduction

Wheat is the leading cereal grain produced, consumed and traded in the world today (Oleson, 1994). It is non-perishable, easy to store and transport, has a good nutritional profile and allows the manufacturing of a wide variety of products. The unique protein composition of wheat makes it suitable for the production of bread.

In wheat bread-making, flour, water, salt, yeast and/or other micro-organisms, often with the addition of non-essential ingredients, such as fat and sugar, are mixed into a viscoelastic dough, which is fermented and baked (Goesaert *et al.*, 2005). Today enzymes play a central role in bread-making and different quality aspects, e.g. flavour, bread volume, crumb structure and shelf-life can be improved with enzymes (Linko *et al.*, 1997).

In this literature study, wheat, the composition of wheat flour, wheat flour quality tests, rheological tests, bread-making (ingredients, additives and processes) and bread staling will be reviewed. The classification, function, specificity and the optimum conditions of three important enzymes in bread-making will be discussed. This will be followed by the role of enzymes in bread-making and staling.

### 2. Wheat

The cultivation of wheat (*Triticum* spp.) is thought to have begun about ten thousand years ago (Figoni, 2004). It is a hardy crop and can be cultivated under a wide range of environmental conditions (Atwell, 2001). Well-known wheat-producing regions include the Ukraine, Buenos Aires Province of Argentina, the lowlands of Europe, the south eastern and south western states of Australia, the Great Plains of the USA and Canada (Atwell, 2001). The total world grain production amounts to *ca.* 2272 million tonnes per annum. Maize, wheat and rice together represent approximately 85% of the total world grain production, with each of them contributing to similar extent (Dornez, 2007). Approximately 75% of the wheat produced worldwide is used for human consumption, while *ca.* 15% is used for feed and the remaining 10% is used for seed and industrial applications (Carter, 2002).

The principal wheats of commerce are cultivars of the species *Triticum aestivum*, *Triticum durum* and *Triticum compactum* (Kent & Evers, 1994). Wheat buyers and sellers have used various systems over the years to come to an agreement on the price to be paid for wheat based on its quality (Halverson & Zeleny, 1988).

The USA is one of the largest producers of wheat and their wheat is exported regularly (Oleson, 1994). In the USA wheat is normally divided into two quality classes, based on its suitability for

bread baking, i.e. hard and soft. Hard wheat has a physical hard kernel that yields flour with high gluten and consequently high protein content suitable for producing a western style loaf of bread and some types of noodles. Soft wheat is characterised by lower protein content and is most suitable for producing biscuits and cakes which do not require strong flour. Semi-hard wheats have some of the above mentioned quality characteristics and are utilised in Asian steamed bread and certain noodles.

Colour (red or white) refers to the colour of the aleurone layer of the wheat kernel. Wheat is also classified according to cultivation habits, i.e. whether it is a spring or winter harvest. In order to provide wheat with consistent quality it is also divided into grades (Oleson, 1994). The grades (U.S. No. 1–5) are based on the purity of the wheat (i.e. percentage contamination by other wheat or grains), percentage of damaged or defective kernels and the presence of foreign material (Atwell, 2001). The grain is also graded according to attributes such as weight, protein and moisture contents (Oleson, 1994).

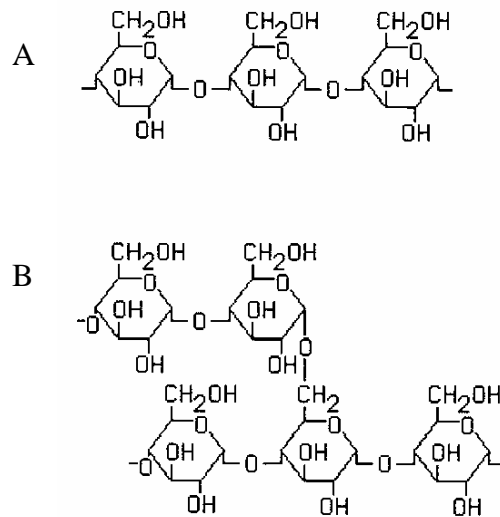
Other major exporters also categorise wheat into classes and grades. In Argentina, there are two major wheat types and four grades (Atwell, 2001). Australia categorises wheat into seven wheat types from seven areas and three major grades. Canada categorises wheat into seven classes and 19 grades. In South-Africa there are four classes of wheat and each class can be divided into different grades according to quality parameters and the percentage of deviations (S.G. Ybema, Wheat Grading Coarse, South Africa, personal communication, 2007).

### 3. Wheat flour composition

The major constituents of wheat flour are starch (70-75%), water (14%) and proteins (10-12%) (Goesaert *et al.*, 2005). Non-starch polysaccharides (2-3%), in particular arabinoxylans and lipids (2%), are important minor flour constituents relevant to bread production and quality.

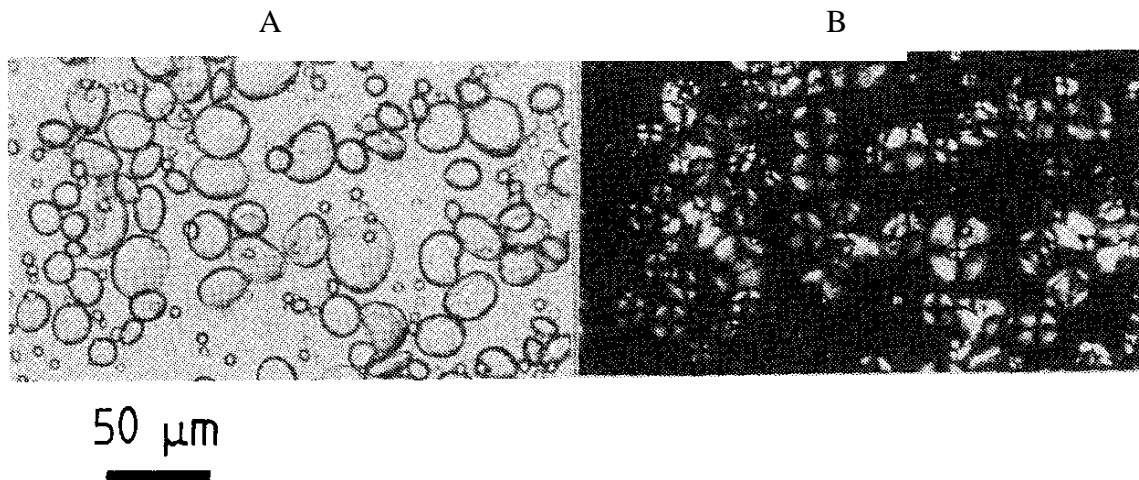
#### 3.1 Starch

**Starch** is the most important reserve polysaccharide and the most abundant constituent of many plants, including cereals (Goesaert *et al.*, 2005). Starch consists of  $\alpha$ -(1,4)-linked D-glucopyranosyl units (Goesaert *et al.*, 2005). The major components are amylose (25-28%) and amylopectin (72-75%). Amylose is slightly branched with  $\alpha$ -(1,6)-linkages (Shibanuma *et al.*, 1994) and consists of more than 2000 glucose units with a molecular weight ranging from 250 000 to 1.9 million (**Fig. 2.1**) (Chung & Park, 1997). Amylopectin is a large, highly branched polysaccharide and each branch consists of 20-30 glucose units with a molecular weight ranging from 1 million to 100 million.



**Figure 2.1** The structure of amylose (A) and amylopectin (B) (Eliasson & Larsson, 1993).

Native wheat starch is birefringent when viewed in polarised light; the birefringence indicates there is some kind of organisation in the starch granule (**Fig. 2.2**) (Eliasson & Larsson, 1993). X-ray diffraction patterns also indicate that the starch granule is partly crystalline. A considerable fraction of starch granules (*ca.* 8%) is mechanically damaged during milling which can severely affect starch properties (Hoseney, 1994). Damaged starch granules have lost their birefringence, have higher water absorption and are more susceptible to (fungal) enzymatic hydrolysis (Hoseney, 1994).



**Figure 2.2** Wheat starch granules observed under the light microscope using ordinary light (A) and polarised light (B) (Eliasson & Larsson, 1993).

At room temperature, starch granules can absorb up to 50% of their dry weight of water, but will swell to a limited extent only (French, 1984). Below a specific temperature (the gelatinisation temperature) this process is reversible. As starch gelatinises, it competes with other components for the available water in the system (Chung & Park, 1997). When the starch suspension is heated above the gelatinisation temperature it undergoes a series of reactions that lead to the irreversible

destruction of the molecular order of the starch granule (Atwell *et al.*, 1988). These changes included loss in birefringence, loss of X-ray diffraction pattern, absorption of water and swelling, change in shape and size of starch granules, leaching of amylose from the granules and the formation of a gel or a paste (Eliasson & Larsson, 1993).

There are several ways to measure the gelatinisation temperature range, but differential scanning calorimetry (DSC) has evolved as the preferred method (Eliasson & Larsson, 1993). A loss in birefringence and crystallinity can be monitored by DSC. Gelatinisation begins at 45°C, reaches a peak at 60°C and is completed at 75°C (Eliasson & Gudmundsson, 1996). At temperatures above 75°C, swelling and leaching continues and a suspension of swollen, amorphous starch granules and solubilised macromolecules or starch paste is formed.

Starch is present in the native state (in the dough) where it appears as semi-crystalline granules (Hug-Iten *et al.*, 1999). During **baking**, when the starch suspension is heated, granules absorb more water and swell although granular identity is retained (Hug-Iten *et al.*, 1999). A small amount of starch (mainly amylose) leaches out into the intergranular phase. Due to phase separation, amylose and amylopectin are not homogeneously distributed in the granules. Part of the solubilised amylose forms inclusion complexes with both added and endogenous lipids, which can be seen by the V crystal type of fresh bread crumb.

Solubilised amylose forms a continuous network upon **cooling**, in which swollen and deformed starch granules are embedded and interlinked (Eliasson & Larsson, 1993). During this time, the starch polysaccharides re-associate to a more ordered or crystalline state known as retrogradation (Atwell *et al.*, 1988) which can be observed as a B type X-ray diffraction pattern (Goesaert *et al.*, 2005).

Bread gradually loses its freshness and stales during **storage**. The staling process comprises several aspects, i.e. loss of moisture and flavour, the crust toughens and crumb becomes more firm and less elastic (Hoseney, 1994). Bread staling is often evaluated by measuring crumb firmness, but this property is also influenced by loaf volume and crumb structure (Gray & Bemiller, 2003). Amylose is considered to have little contribution to crumb firming, because it is almost completely retrograded in the bread after cooling (Hug-Iten *et al.*, 1999). The firming of the crumb during aging is mainly attributed to amylopectin retrogradation (Gray & Bemiller, 2003).

### 3.2 Proteins

**Proteins** can be divided into albumins (extractable in water), globulins (extractable in diluted salt solutions), gliadins (extractable in aqueous alcohols) and glutenins (extractable in diluted acetic acid solutions) based on their solubility (Osborne, 1924). Wheat proteins can be divided into two

groups, non-gluten proteins (with either no or just a minor role in bread-making) and gluten proteins (with a major role in bread-making) (Goesaert *et al.*, 2005).

The non-gluten protein fraction consists of about 60% albumins and 40% globulins, peptides, amino acids, flour enzymes, soluble and foaming proteins as well as coagulable proteins (Chung & Park, 1997). Most non-gluten proteins are metabolic (mainly enzymes) or structural proteins. Although it is generally accepted the gluten fraction determines the bread-making potential of wheat flour, some non-gluten proteins may also play a role. Several endogenous wheat enzymes (such as proteases and endoxylanases) and enzyme inhibitors (such as protease inhibitors and xylanase inhibitors) have the potential to affect bread-making performance (Veraverbeke & Delcour, 2002).

**Gluten** proteins are the major storage proteins in wheat. They belong to the prolamin class of seed storage proteins (Shewry & Halford, 2002). The gluten fraction consists of about 45% **gliadins** and 55% **glutenins** (Chung & Park, 1997). **Gliadins** are small, non-polymeric, monomeric proteins with a molecular weight in the range of 30 000-80 000 and are classed into three types,  $\alpha$ -gliadins,  $\gamma$ -gliadins and  $\omega$ -gliadins (Veraverbeke & Delcour, 2002). **Glutenins** are large, polymeric proteins that consist of subunits linked via disulphide bonds. These glutenin subunits can be liberated by reduction of disulfide bonds by reducing agents. Four different groups of glutenin subunits can be distinguished, i.e. high molecular weight (HMW) glutenins with a molecular weight of between 65 000-90 000 and B-, C- and D-type low molecular weight (LMW) glutenins with a molecular weight of between 30 000-60 000 (Veraverbeke & Delcour, 2002).

During dough **mixing** flour is hydrated and because of mechanical energy input the gluten proteins are disrupted. Gluten proteins are transformed into a continuous cohesive viscoelastic gluten protein network (Singh & MacRitchie, 2001). During mixing the resistance of the dough increases, reaches an optimum and finally decreases (over mixing). This can be monitored with a Farinograph and a Mixograph. Carbon dioxide is produced during **fermentation** and the initial stages of baking; with the gluten protein network playing a major role in retaining the carbon dioxide while the dough is fermenting. Glutenins provide strength (resistance to deformation) and elasticity (Belton, 1999; Ewart, 1972) while gliadins provide extensibility in wheat flour doughs (Cornec *et al.*, 1994). Therefore, an appropriate balance between the glutenins and the gliadins are required. During **baking**, changes in protein surface hydrophobicity, sulphhydryl/disulphide interchanges and formation of new disulphide cross-links occur (Jeanjean *et al.*, 1980). The typical foam structure of baked bread is formed as a result of the heat induced changes as well as the changes in the starch (Goesaert *et al.*, 2005). The role of gluten proteins during **staling** of bread is still not clear, but it is generally believed that starch-gluten interactions are somehow involved in bread firming (Gray & Bemiller, 2003).

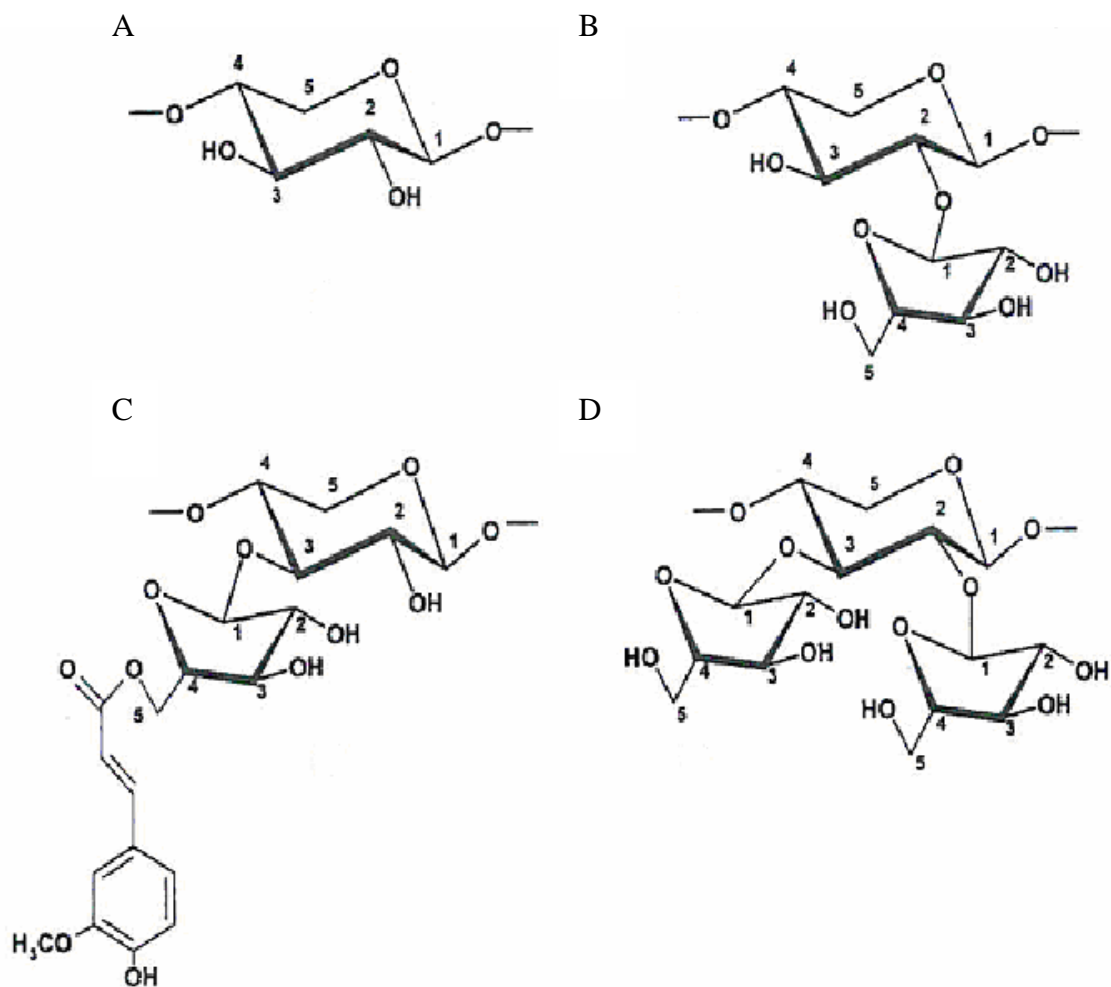
### 3.3 Non-starch polysaccharides

The endosperm and aleurone cell walls of many cereals contain **non-starch polysaccharides** (NSP) which can be divided into cellulose,  $\beta$ -glucans, lignin, pectic substances and pentosans or hemicelluloses (Hille & Schooneveld-Bergmans, 2004; Eliasson & Larsson, 1993). The pentosans or hemicelluloses encompass the non-starch and non-cellulosic polysaccharides of plants including arabinoxylans (AX), arabinogalactans and glucomannans (Hoseney, 1986). Up to 75% of dry weight of wheat endosperm cell walls consists of NSP of which AX are by far the most prominent group (85%) (Goesaert *et al.*, 2005). Although wheat endosperm AX can be divided into two polydisperse groups, i.e. water-extractable arabinoxylans (WE-AX) and water-unextractable arabinoxylans (WU-AX), one general structure applies (Courtin & Delcour, 2002). Arabinoxylans are made up of a backbone of  $\beta$ -1,4-linked D-xylopyranosyl residues, which can be substituted at the C(O)-3 and/or the C(O)-2 position with monomeric  $\alpha$ -L-arabinofuranoside (Perlin, 1951a,b). Ferulic acid can be coupled to the C(O)-5 of arabinose through an ester linkage (Fausch *et al.*, 1963). This results in four basic building blocks: unsubstituted xylose residues, C(O)-2-monosubstituted xylose, C(O)-3-monosubstituted xylose and C(O)-2- and C(O)-3-disubstituted xylose (**Fig. 2.3**) (Courtin & Delcour, 2002).

The physico-chemical properties of AX (solubility, cross-linking and gelation, foam stabilisation, viscosity and water holding capacity) are mainly determined by the following parameters: the length of the xylan backbone; the degree of substitution or A/X ratio; the substitution pattern; and the binding of ferulic acid to other AX or cell wall components (Courtin & Delcour, 2002).

It has been reported the addition of pentosans improved the loaf volume and crumb quality of bread (Cawley, 1964; Jelaca & Hlynka, 1972). In contrast, Kim and D'Appolonia (1977a) demonstrated the addition of water-soluble pentosans did not affect the loaf volume, but the addition of water-insoluble pentosans slightly decreased loaf volume. The WE-AX have a strong influence on viscosity of the aqueous medium and make up 20-25% of the total AX content. In contrast to the WE-AX, the WU-AX have a strong water holding capacity. They can bind up to ten times their weight in water and may contribute approximately to one third of the water-binding capacity of dough. Their insoluble nature is due to covalent and non-covalent interactions with adjacent AX, protein and/or cellulose molecules (Vardakou *et al.*, 2003). When WE-AX were added to flour, two thirds of the intrinsic viscosity of flour extracts was attributed to the WE-AX (Udy, 1956). Under oxidising conditions they cross-linked by covalent coupling with two ferulic acid residues (Figueroa-Espinoza & Rouau, 1998) and this caused a strong increase in viscosity of AX solution (Izydorczyk *et al.*, 1990). The WE-AX stabilises protein films against thermal disruption, but lowers initial foam formation.





**Figure 2.3** Non-substituted D-xylopyranosyl-residue (A), D-xylopyranosyl residue substituted on C(O)-2 with a L-arabinofuranosyl residue (B), D-xylopyranosyl residue substituted on C(O)-3 with a L-arabinofuranosyl residue (C), D-xylopyranosyl residue substituted on C(O)-2 and C(O)-3 with L-arabinofuranosyl residues (D). Structure C shows the link of ferulic acid to C(O)-5 of a L-arabinofuranosyl residue (Courtin & Delcour, 2002).

The addition of WE-AX to flour can result in several positive effects on the **dough** including: an increase in dough consistency, stiffness, resistance to extension (Jelaca & Hlynka, 1972) and water absorption (Biliaderis *et al.*, 1995); as well as a decrease in mixing time (Jelaca & Hlynka, 1972), energy input needed to achieve optimal mixing (Jelaca & Hlynka, 1971) and extensibility (Jelaca & Hlynka, 1972). The addition of WU-AX to flour has the same effect as WE-AX, except for dough extensibility (Goesaert *et al.*, 2005). According to Goesaert *et al.* (2005), the mechanisms by which AX affect the initial stage of baking are similar to those observed for fermentation.

The addition of WE-AX decreases the diffusion rate of carbon dioxide during **fermentation**, leading to better gas retention. Increased dough foam stability leads to an increase in viscosity of

the dough and in return stabilises gas cell liquid films. It has been postulated the WU-AX might have a negative impact since they will destabilise the dough structure, by forming physical barriers preventing gluten network formation during dough development. They also absorb a large amount of water that is not available for gluten development and film formation, and/or perforate gas cells which will coalesce (Courtin & Delcour, 2002).

The WE-AX will stabilise gas cells during **baking**, which will prolong oven rise and improve bread characteristics (Goesaert *et al.*, 2005). The WU-AX will enhance gas cell coalescence and decrease gas retention (Courtin & Delcour, 2002).

It has been reported that pentosans decreased retrogradation of starches and staling of bread. This is based on lower firmness values observed for starch gels and bread containing pentosans (Kim & D'Appolonia, 1977b; Jankiewicz & Michniewicz, 1987). The presence of AX may interfere with starch intermolecular associations and therefore, may lower retrogradation (Kim & D'Appolonia, 1977a,b). Others attribute the effect of AX on bread staling mainly to their strong effect on water distribution in the dough (Biliaderis *et al.*, 1995).

On a commercial scale, it is at present, not possible to change dough properties through AX addition, due to the lack of industrial feasible AX isolation procedures and therefore the lack of commercial AX products. However, the AX functionality in bread-making can be optimised by using microbial derived endoxylanase (E.C. 3.2.1.8) (Goesaert *et al.*, 2005).

### 3.4 Lipids

The total lipid content of wheat flour is 2.5-3.0% (Chung & Park, 1997). In the wheat kernel, the germ has the largest amount of lipids and these lipids contain the highest percentage of phospholipids. In cereal literature, **lipids** are often defined as **free** or **bound**; this distinction is based upon solubility. The **free lipids** are easily extractable with a non-polar solvent such as petroleum ether or hexane. **Bound lipids** are extractable with polar solvents such as an aqueous mixture of alcohol at ambient temperatures. Another important distinction is that of **polar** and **non-polar lipids**. **Non-polar lipids** include hydrocarbons, free fatty acids and triglycerides. **Polar lipids** include phospholipids and glycolipids. In wheat flour, the lipid content can be divided into the lipids associated with starch granules and non-starch associated lipids (Hoseney, 1994). Non-starch associated lipids make up one third of total lipids (Chung & Park, 1997) and can be divided into about 60% non-polar lipids and 40% polar lipids. The starch associated lipids make up two thirds of total lipids (Chung & Park, 1997) and can be divided into 90% polar lipids and 9% non-polar lipids. They are extractable with polar solvents at an elevated temperature (Chung & Park, 1997). Starch associated lipids are strongly bound in the starch granules which make them essentially unavailable to effect dough processing before gelatinisation occurs (Eliasson & Larsson, 2003).



The incorporation of lipids into bread dough results in a larger, final loaf volume, improved oven spring, a softer crumb, a less crisp crust as well as an improved keeping quality of the bread (Stauffer, 1993). The use of lipases (E.C. 3.1.1.3) in breadmaking is quite recent when compared to that of other enzymes (Qi Si, 1997). Lipases hydrolyse the ester bonds of mainly the triglycerides, yielding mono- and diglycerides as well as free fatty acids. The 1,3-specific lipases (which remove fatty acids from the 1- and 3- positions) in particular, improve dough rheological properties as well as the quality of the baked product and may be used as an alternative to chemical dough strengthening emulsifiers. The use of lipases in the baking industry has escalated, especially the use of lipases with activity towards polar lipids (Erlandsen *et al.*, 2007). Besides improving bread volume, crumb structure and crumb softness, these lipases provide a dough stabilising effect and they are used as cost efficient alternatives to emulsifiers in many bread processes. It was found that lipases with activity towards polar lipids are more efficient in promoting changes to surface properties than lipases only active on triglycerides (Erlandsen *et al.*, 2007).

### 3.5 Enzymes

Enzymes such as  $\alpha$ - and  $\beta$ -amylases, proteases, lipases, esterases, phytases, oxidases and peroxidases are naturally present in wheat flour (Kent & Evers, 1994). Arabinoxylan degrading enzymes (endoxylanases) are endogenously present in a number of cereals, such as wheat, barley and rye (Cleemput *et al.*, 1995). The endoxylanases are not homogeneously distributed in the wheat kernel, but are present in different tissues (Bonnin *et al.*, 1998). The bran and shorts fractions contain much higher activity levels than flour fractions (Schmitz *et al.*, 1974; Bonnin *et al.*, 1998).

## 4. Wheat flour quality tests

It is important to know the quality of the flour before it is used to produce products such as bread. The quality of the flour can be determined by chemical analysis on the flour and rheological analysis on the dough. The results will enable the production of a consistent end-product.

### 4.1 Protein content

Protein is probably the most important determinant in bread flour quality (Hoseney, 1986). Cereal grains vary widely in their chemical composition and this variation is also noticeable in their protein content. Wheat contain from less than 6% to more than 27% protein, with most commercial samples containing 8 to 16% protein. The wide variation is a result of the combination of environmental conditions and genetic background of the wheat (Hoseney, 1986).

In wheat, the crude protein can be determined using either the Kjeldahl (AACC Approved Method 46-10; AACC, 2000 and ICC Standard No. 105/2; ICC, 2008) or Combustion methods

(AACC Approved Method 46-30; AACC, 2000 and ICC Standard No. 167; ICC, 2008). Kjeldahl analysis includes sample digestion in boiling sulphuric acid, neutralisation with sodium hydroxide solution, distillation of the resulting ammonia gas into a trapping solution, titration with an acid solution and finally, determination of the amount of nitrogen and protein by calculation (Anon., 1995). In the Combustion method a sample is burned in an oxygen-rich atmosphere, the amount of nitrogen gas is measured and the total protein present is calculated from the nitrogen content. For wheat bran, whole wheat flour and wheat flour a factor of 5.7 is used to convert the total nitrogen in food to protein in both methods (AACC Approved Method 46-30; AACC, 2000). The protein content of ground wheat and flour can also be estimated by NIR spectroscopy according to ICC Standard No. 159; (ICC, 2008) and AACC Approved Method 39-11 (AACC, 2000). The protein content of whole grain wheat flour can be estimated according to AACC Approved Method 39-25 (AACC, 2000).

#### *4.2 Moisture content*

Moisture content is another important consideration in determining the quality of wheat. Moisture content in wheat has direct economic importance because it is inversely related to the amount of dry matter in wheat. Even more important is the effect of moisture on the keeping quality of wheat (Halverson & Zeleny, 1988).

According to the AACC Approved Method 44-15A; (AACC, 2000) and ICC Standard No. 110/1; (ICC, 2008) the steps involved in determining moisture content include: weighing the moisture dishes and their lids, weighing and adding a specific amount of the sample and placing this in an air oven or drying cabinet at 130°C for 60 minutes. The samples are then closed and transferred to a desiccator for 45-60 minutes to cool to room temperature before being weighed. The moisture content of the samples is determined as the difference between the weights of the original sample and the dried sample (AACC Approved Method 44-15A; AACC, 2000). The moisture content of ground wheat and the products of wheat milling can also be estimated by NIR spectroscopy according to ICC Standard No. 202; (ICC, 2008).

#### *4.3 Hagberg Falling Number*

The occurrence of rainy weather after wheat has matured in the field, but before it is harvested, may cause some of the kernels to sprout. Such kernels display high levels of  $\alpha$ -amylase activity. Even before sprouting becomes visible, the  $\alpha$ -amylase activity level might have been elevated considerably. Increased proteolytic activity normally accompanies the increased  $\alpha$ -amylase, active in sprouting wheat, and may have a negative effect on bread baking quality (Halverson & Zeleny, 1988). Falling Number is an empirical test based on the ability of the endogenous  $\alpha$ -amylase to

reduce the viscosity of heat-treated flour slurries (Poutanen, 1997). This method is widely used in the baking and milling industry to estimate the baking quality of flour.

The test records the time in seconds it takes a stirrer to stir and to allow a viscometer-stirrer to fall a fixed distance through a hot aqueous flour suspension being liquefied by the enzyme in a standardised apparatus (AACC method 56-81B; AACC, 2000 or ICC Standard No. 107/1; ICC, 2008). The sample is weighed (7.00 g on moisture basis content), placed in a viscometer tube and water (25 mL) is added. The water will activate the  $\alpha$ -amylases present in the flour which will commence on degrading the starch. This mixture is shaken ( $40 \pm 10$  times) and placed in a water bath ( $90^\circ\text{C}$ ) where the starch begins to gelatinise (Anonymous, 1997). This method is applicable to both meal and flour of small grains as well as to malted cereals (AACC method 56-81B; AACC, 2000).

#### 4.4 Ash determination

Both the crude fibre and the ash content of wheat are related to the amount of bran in the wheat. Small or shrivelled kernels may have more bran (on a percentage basis) and therefore more crude fibre and ash than large, plump kernels. They also consequently yield less flour. Wheat usually contains 2.0-2.1% crude fibre and 1.4-2.0% ash calculated on a 14% moisture basis (Halverson & Zeleny, 1988). The total ash content (AACC method 08-01/2; AACC, 2000 or ICC Standard No. 104/1; ICC, 2008) of flour and grain samples is measured by placing the samples in a muffle furnace at  $700^\circ\text{C}$  for 3 hours, after which the samples are left to cool in a desiccator and the percentage of ash is determined (James, 1996). The ash content of the flour can be significantly affected by a small amount of bran present and mills are compelled to produce flours with small bran content as specified by customers (AACC method 08-01/2; AACC, 2000).

#### 4.5 Colour

It is important to distinguish between the light yellow or creamy colour contributed to bread crumb by the pigments of the wheat and the grey cast and dull appearance contributed by the presence of bran in high-extraction flours (Kruger & Reed, 1988). The yellowish wheat pigments that are extractable with organic solvents are usually referred to as carotenoids because the provitamin A, carotene, had first been (incorrectly) reported as the principal pigment (Kruger & Reed, 1988). Carotene is easily oxidised and the bleaching agents used in the milling industry destroy the provitamin. Quantitation is based on measurement by instruments that determine the reflectance of flour or flour pastes (Kruger & Reed, 1988). The Kent-Jones and Martin flour grader has been widely used for this purpose. When determining the colour, a paste is prepared from a wheat flour sample and contained in a glass cell (Kent-Jones *et al.*, 1956). The test is based on the measurement of the reflection of light (in the 492-577 nm wavelength regions) from the surface of a paste in the

glass cell with the results expressed on an empirical scale. The use of these conditions results in a measurement known as the Grade Colour which greatly influences the consumers' degree of preference of a product.

#### *4.6 Sedimentation test*

The sedimentation test for wheat and wheat flour (AACC method 56-60/61A; AACC, 2000 or ICC Standard No. 116/1; ICC, 2008) measures the relative gluten strength in wheat flour, because gluten proteins have the ability to swell under the influence of lactic acid. Sedimentation volumes (values) reflect differences in both protein quantity and protein quality. A positive correlation has been established between sedimentation volume and gluten strength or loaf volume. The method is used as a screening test in wheat breeding. In commercial or experimental milling, the sedimentation test is usually used for comparing a lot of the same grade of flour milled by the same mill (AACC method 56-60/61A; AACC, 2000).

### **5. Rheology**

Rheology is the study of the flow and deformation of materials (Dobraszczyk & Morgenstern, 2003). The study of the rheological properties of materials involves imparting work by means of shearing, stretching or compressing and interpreting the resulting stress-strain-time relationships in terms of elastic and viscous properties. Many materials exhibit both elastic and viscous properties and are termed viscoelastic materials. These materials can be further subdivided into linear and non-linear viscoelastic materials. For linear viscoelastic materials the ratio of stress to strain is a function of time alone and not of the stress magnitude. For non-linear viscoelastic materials, the ratio of stress to strain is a function of stress magnitude in addition to time. Wheat flour dough can be classified as non-linear viscoelastic material; its elastic and viscous properties during stress are dependent upon both the time of stress and stress magnitude (Shuey, 1984). Within the cereal industry there has been a long history of using descriptive empirical measurements of rheological properties, using a range of devices such as, the Penetrometer, Texturometer, Consistograph, Amylograph, Farinograph, Mixograph, Extensigraph and Alveograph (Dobraszczyk & Morgenstern, 2003).

In **bread-making**, the dough undergoes some type of deformation in every phase of the process. During **mixing**, the dough undergoes extreme deformations beyond the rupture limits; during **fermentation** the deformations are much smaller; during **sheeting** and **moulding**, the deformations are of an intermediate level; and finally during **proofing** and **baking**, the dough is subject to more deformations. The results obtained from devices which measure the rheological properties of dough can be used to predict how the dough will behave during further processing. These tests are very

useful when new ingredients are evaluated as the results will predict how the ingredients will affect the dough or bread quality. When flour does not meet the required specifications, these results can be used as guidelines to adjust the process parameters.

### *5.1 Alveograph*

The Alveograph measures resistance of the dough to extension and the extent to which it can be stretched under the conditions of the method (AACC Approved Method 54-30A; AACC, 2000 or ICC Standard No. 121.A; ICC, 2008). Dough with a definite thickness is prepared under specific conditions and expanded by air pressure until it ruptures. The internal pressure in the bubble is graphically recorded (AACC Approved Method 54-30A; AACC, 2000). Unlike the Extensigraph that stretches the dough in one direction (uniaxial), the Alveograph stretches the dough at equal rates in two directions, referred to as biaxial extension (Dobraszczyk & Morgenstern, 2003). Properties such as strength (S, cm<sup>2</sup>), stability (P, mm), distensibility (L, mm), deformation energy (W, x10<sup>-4</sup>J), P/L ratio and swelling index (G) is determined by the Alveograph (Faridi & Rasper, 1987).

The area under the curve is an indication of the strength of the dough and is measured in cm<sup>2</sup> (Faridi & Rasper, 1987). It reflects the ability of the dough to retain gas during fermentation and baking. To obtain the stability of the dough, the maximum height of the graph is measured and multiplied by 1.1 which gives an indication of the resistance of the dough against extension. The stability is measured in mm. To obtain the distensibility of the dough the length of the curve is measured from the point where the bubble inflates to the point where it bursts. It is measured in mm and it is an indication of the extensibility of the dough and also predicts the handling properties of the dough. The deformation energy of the dough is the energy required to inflate the dough until it ruptures. It is related to the baking strength of the flour. The P/L ratio is obtained by dividing the stability by the distensibility and this combines the values for dough stability and dough extensibility (Faridi & Rasper, 1987).

### *5.2 Consistograph*

When conducting a consistograph test a dough is made from wheat flour to which an amount of water (based on the initial moisture content of the flour) is added in order to reach a constant hydration level on a dry-matter basis (AACC Approved Method 54-50; AACC, 2000). During the kneading of this dough sample, the pressure on one side of the mixer is continuously monitored. The peak pressure (Prmax, mb) recorded during kneading is used to calculate the water absorption (at 14% and 15% moisture basis) of the flour sample at a given consistency (equivalent to a pressure of 1700 mb). Physical properties of the wheat flour dough are determined in a subsequent

test performed at the hydration level previously determined (AACC Approved Method 54-50; AACC, 2000).

### *5.3 Farinograph*

The Farinograph employs broad, sigmoid shaped paddles and measures dough consistency as it is kneaded between two of these blades (AACC Approved Method 54-21; AACC, 2000 or ICC Standard No. 115/1; ICC, 2008). The motor driving the blades is not fixed, but moves within a limited arc on its rotational axis (Ingelin, 1997). The Farinograph measures and records the resistance of the dough to mixing. It is used to evaluate water absorption of flour and to determine stability and other characteristics of dough during mixing.

There are two basically different procedures, i.e. constant flour weight and constant dough weight (AACC Approved Method 54-21; AACC, 2000). Properties such as water absorption (at 14% moisture basis), dough development time (min), stability (min), mixing tolerance index (MTI) and degree of softening are determined by the Farinograph (Preston & Kilborn, 1984). Water absorption gives an indication of the amount of water to add during bread-making. The stability of the dough gives some indication of the flour's tolerance to mixing and the dough development time gives an indication of the optimum mixing time of the dough.

### *5.4 Mixograph*

The Mixograph has two pairs of relatively thin pins rotating in a planetary motion and has three fixed pins in the mixing bowl to oppose the action of the moving pins (AACC Approved Method 54-40A; AACC, 2000). The mixing bowl is free to a limited degree in response to torque transmitted through the dough (Ingelin, 1997). The mixograph measures and records the resistance of the dough to mixing. The mixing curve indicates optimum development time (min), tolerance to overmixing and also estimates water absorption (%). The mixograph can be used to study the effects of added ingredients on mixing properties, dough rheology and blending. It can also be used for quality control and the evaluation of hard, soft and durum wheat (AACC Approved Method 54-40A; AACC, 2000).

## **6. Bread-making**

The aim of all bread-making processes is essentially the same, namely to convert wheat flour and other ingredients into a light, aerated and palatable food. Bread is probably the oldest processed food and its discovery most likely took place in the Middle East (Cauvain & Young, 2006). Bread is an unstable, elastic, solid foam with the solid part containing a continuous phase which is composed of an elastic network. This elastic network is formed by cross-linked gluten molecules and leached

starch polymer molecules (primarily amylose), together with polar lipid molecules. There is also a discontinuous phase of entrapped, gelatinised, swollen, deformed starch granules (Gray & Bemiller, 2003). The basic ingredients for bread-making are flour, water, yeast, sugar, salt and shortening (Chung & Park, 1997). The bread baking process has recently been reviewed by Mondal and Datta (2008).

### 6.1 Flour

To make any bread product, the flour specification must meet the product and process requirements (Brown, 1993). Protein quality and quantity, water absorption,  $\alpha$ -amylase activity and starch damage have to be matched with product and process requirements, as well as with the influence of other ingredients in the recipe (Brown, 1993). In a recipe, flour is always 100%, while the rest of the ingredients are a percentage of that amount by weight. Based on 100% flour, the rest of the ingredients will usually be added in the following amounts: yeast (3%), sugar (2%), salt (2%) and shortening (3%) (Mondal & Datta, 2008).

### 6.2 Water

Water and flour are the most significant ingredients in a bread recipe as they affect texture and crumb the most (Mondal & Datta, 2008). Water represents nearly 40% of the dough mass and 35% of the bread mass (Brown, 1993). Water serves as a medium for physical, chemical, biochemical and biological reactions and by allowing various interactions during bread-making, raw materials can be changed and combined into a finished baked bread. Water is added to hydrate flour proteins and to be partially absorbed by the flour starch (particularly the damaged starch). It also forms a water phase in the dough in which soluble solids such as sugars, salts and soluble proteins are dissolved and in which yeast cells are dispersed (Brown, 1993). When water is first added to flour, rapid hydration of flour particles occurs that results in the formation of a fine dough film. As mixing continues, water performs as a plasticiser (Chung & Park, 1997). Approximately 50% added water results in a finely textured, light bread. In yeast breads, the higher water percentages result in more carbon dioxide bubbles and a coarser bread crumb (Mondal & Datta, 2008).

### 6.3 Yeast

The yeast used for bread-making is a variety of the species *Saccharomyces cerevisiae* (Sluimer, 2005). Under aerobic conditions, this yeast metabolises glucose with the release of carbon dioxide, but under anaerobic conditions, carbon dioxide and ethanol is produced through fermentation (Sluimer, 2005). Commercial forms of yeast can be divided into two main classes, i.e. fresh yeast and dried yeast. Fresh yeast is available as compressed yeast, granular yeast and bulk liquid or



cream yeast. The moisture content of compressed and granular yeast is about 72% whereas bulk liquid or cream yeast has a moisture content of 82%. Fresh yeast is a perishable product (due of yeast autolysis) and therefore handling and storage should take place at temperatures between 0 and 4°C. Dried yeast is available in two commercial forms: active dry yeast and instant dry yeast. Active dry yeast has a moisture content of 8% and produces much lower leavening activity than fresh yeast. Instant dry yeast has a moisture content of about 5% and the activity is near that of compressed yeast (Sluimer, 2005).

The commonly used yeast concentration in bread-making is 3.5-5.0% of flour weight for either compressed or bulk yeast and 0.8-1.2% for active dry or instant dry yeast (Chung & Park, 1997). The main functional role of yeast in bread-making is its leavening effect to convert fermentable carbohydrates to produce carbon dioxide and ethanol through anaerobic fermentation. The yeast cells take up nutrition in liquid form through its cell walls and produces carbon dioxide and ethanol as by-products of this metabolism. The carbon dioxide goes into solution in the dough/water phase, which when saturated with carbon dioxide, releases it into gas cells in the dough which have been formed during mixing and processing (Brown, 1993). The production of carbon dioxide is imperative to obtain a loaf of bread with the desired volume and a light crumb texture. The yeast also produces flavour precursors that contribute to flavour development (Chung & Park, 1997).

#### *6.4 Sugar*

Sucrose in dough is hydrolysed, almost instantly, to glucose and fructose by the yeast invertase enzyme resulting in all the sucrose being hydrolysed by the end of the mixing period (Chung & Park, 1997). Sugar also serves as a nutrient for yeast, being fermented into carbon dioxide, alcohol and other minor components. The carbon dioxide is the major contributor to loaf volume and crumb texture. The sugar level for adequate carbon dioxide production can be maintained with 2% sugar in the sponge and dough process and 3% sugar in the straight-dough process (**See 6.11 on Bread-making processes**). Sugars that remain unfermented by the yeast appear as residual sugars in the finished bread where they exert significant effects on product quality. These residual sugars also enhance bread flavour and aroma through the development of volatile acids and aldehydes (Chung & Park, 1997)

The main features influencing consumer preference is bread surface colour, texture and flavour (Purlis & Salvadori, 2007). The yellow-gold colour formation is often called browning and is caused by chemical reactions such as caramelisation and Mallaird reaction. Caramelisation takes place when carbohydrates are heated directly, while the requirements for Mallaird browning are the presence of an aminic compound (usually a protein), a reducing sugar and some water.



Certain sweeteners, such as levulose, honey and invert sugar can extend the shelf-life of a product by increasing the moisture retention due to their hygroscopic nature. The sugar also acts as a stabiliser and sweetener (Hoseney, 1986).

### *6.5 Salt*

The salt level normally used in a bread formula is in the range of 1.5-2.0% of flour weight (Chung & Park, 1997). Salt is used in bread formulations not only to impart flavour, but also to increase dough strength; usually seen as an increase in mixing time. The pH of dough affects mixing time by influencing the charge of the proteins, with lower pH giving a shorter mixing time. Salt increases dough strength and tightens it by shielding charges on the dough proteins (Hoseney, 1986). Salt has been shown to have further flavour enhancing effects such as increasing the perception of sweetness, masking possible off-tastes and most importantly, improving the flavour balance (Chung & Park, 1997). Salt also influences the speed of fermentation in the dough by reducing the yeast activity at certain levels (Sluimer, 2005). Addition of 1% salt (flour basis) reduces the yeast activity by 6%; 2% salt addition leads to a reduction of 20% and 4% salt decreases the yeast activity by about 70%. In many countries, bread salt contains iodide to supplement an insufficient amount of iodide in the diet. Potassium or ammonium chloride is sometimes used to replace sodium chloride, partly or totally. Unfortunately, potassium contributes to a rather bitter taste and ammonium adds a liquorice taste to the end-product.

### *6.6 Shortening*

Shortening (ghee or margarine) is added to increase the machinability of dough or specifically slicability of bread (Mondal & Datta, 2008). During dough mixing, fat crystals develop a crystal-water interface that allows for adsorption to the gas-liquid interface of the bubbles (Brooker, 1996). During this process the interface surrounding each crystal coalesces with the gas-liquid interface of the bubble. The expansion of bubbles during proofing leads to the adsorption of more fat crystals as they are encountered in the aqueous phase. Fat crystals melt during baking, making it possible for the crystal liquid interface to be incorporated into the surface of the bubble as it expands. This transfer of interfacial material from crystal to bubble surface explains how the addition of shortening to dough allows bubbles to expand during baking without rupturing, thus producing bread with high loaf volume and fine crumb structure. According to Chung and Park (1997), loaf volume increases with an increase in the amount of shortening up to a concentration of 3% for a pup-loaf (100 g flour). The overall palatability of the product is improved, the keeping quality of the product is extended and the crumb grain is improved by tenderising and imparting shortness to the crumb structure.

### 6.7 Soyflour

Soybean is a legume used for the production of soymeal. Compared to wheat, dried soybeans have a higher protein (about 35%) and fat (about 20%) content and are lower in starch (15-20%). Untoasted soyflour contains active enzymes that are useful in yeast breads, but only about 0.5% of untoasted soyflour is needed in bread-making. Toasted soyflour does not contain active enzymes and has a more appealing flavour, so it can be used at higher concentrations than untoasted soyflour (Figoni, 2004).

The benefits of full fat, enzyme-active soyflour in bread-making have long been recognised. It improves dough handling properties, stability and gas retention, providing better oven spring, product volume, improves crumb texture and colour. The soyflour enzyme, lipoxygenase has a bleaching effect on flour carotenoids through a series of complex reactions with flour lipids and atmospheric oxygen and can be used to replace chemical bleaching agents (Brown, 1993). Wheat and other cereal grains are low in the essential amino acid lysine, while soy protein has a high lysine content and can therefore be used in breads to improve their protein quality. Soyflour also increases water absorption of dough (Figoni, 2004).

### 6.8 Dried gluten

Dried gluten is widely used as a flour protein supplement (Brown, 1993). The combined benefits of dried gluten and diacetyl tartaric acid esters of mono- and diglycerides (DATEM) (**See 6.10.3 on Emulsifiers**) are used in many products, in particular wholemeal breads and specialist brown and white breads which may contain added fibre, whole grains, softened grains, cracked wheat and seeds. Dried gluten and DATEM provide improved dough stability, gas retention and product volume needed by these products. Gluten is not listed as an additive on the label, but as a wheat protein and therefore it is regularly used in products where the addition of additives has to be limited.

### 6.9 Malt flour and fungal $\alpha$ -amylases

Flour contains an abundant supply of  $\beta$ -amylases, but may have to be supplemented with  $\alpha$ -amylases (Drapron & Godon, 1987). Two sources of supplementation is possible, i.e. enzyme active malt flour (provides cereal  $\alpha$ -amylases) and fungal  $\alpha$ -amylases (Brown, 1993). Both sources of  $\alpha$ -amylases will be satisfactory supplements for flour deficient in  $\alpha$ -amylases and ensure that there is an adequate supply of maltose, if required for yeast metabolism. Flour standardisation is mostly performed by adding fungal  $\alpha$ -amylases from *Aspergillus oryzae* (Bowles, 1996). During baking when the hydrated starch flour gelatinises it becomes available for enzymatic attack (Brown, 1993). The  $\alpha$ -amylases produce dextrins, but excessive amounts of dextrins, results in bread with a

sticky crumb. The action of the amylases (on the gelatinised starch) release water, causing softening of the gelatinised starch structure, which appears to generate a better oven spring. However, excessive dextrin production and softening, leads to excessive oven spring in combination with the weak sticky crumb; causing excessive volume and resulting in the collapse of the sides of the bread. In general, fungal  $\alpha$ -amylases are more heat sensitive than cereal  $\alpha$ -amylases. They are inactivated by the increasing dough temperature before excessive conversion of gelatinised starch to dextrin can take place (Brown, 1993).

#### *6.10 Additives used in bread-making*

The functionality of gluten in wheat flour is determined by the molecular weight of glutenin, the occurrence of covalent and non-covalent bonds between glutenin molecules, and interactions between glutenin and other flour constituents. The most prominent linkages are disulphide bonds that hold the glutenin subunits together. Oxidising and reducing agents, which have a strong impact on the thiol-disulphide system, can affect the polymerisation of glutenin subunits and thereby change the mechanical and rheological properties of the dough (Fitchett & Frazier, 1986).

##### *6.10.1 Reducing agents*

By reducing the disulphide bonds in flour proteins to dissociate sulfhydryl groups, the reducing agent causes the dough to become more flexible and extensible (Hoseney, 1986). Reducing agents, such as L-cysteine and sodium metabisulphite, may be added to weaken the dough structure. By reducing dough resistance to deformation, they help in moulding and shaping without structural changes. L-cysteine can also be used in combination with oxidising agents. During kneading, cysteine cleaves disulphide bonds, which facilitates gluten protein distribution in the dough and improves extensibility. Once a good distribution is achieved, the oxidising agent restores dough strength (Fitchett & Frazier, 1986).

Proteases can also be used to degrade proteins in the gluten network. In contrast to the impact of chemical reducing agents, the peptide bonds and original rheological condition cannot be restored. Proteases can be added to shorten mixing time, to reduce dough consistency, to regulate gluten strength in bread, to assure dough uniformity, to control bread texture and to improve flavour (Mathewson, 2000).

##### *6.10.2 Oxidising agents*

Flour is said to be bromated when potassium bromate is added to flour. Potassium bromate has been used since the 1900s and is the standard against which all other maturing agents are judged. Potassium bromate works primarily during final proofing and oven spring, when strength is needed

most (Figoni, 2004). Potassium iodate and potassium bromate are able to reform some of the intermolecular disulphide bonds of gluten proteins and restore dough strength (Goesaert *et al.*, 2005).

Toxicological studies indicated that bromate could be carcinogenic (Goesaert *et al.*, 2005). Therefore, potassium bromate is no longer allowed as a flour additive in the European Union, Australia, New Zealand and Canada, however, there is still limited use in the USA (Cauvain & Young, 2006). Potassium bromate is not accepted for use in South Africa. Azodicarbonimide is still permitted and often used instead of potassium bromate (D.P. Clayton, Manger Director, Danisco Baking, South Africa, personal communication, 2008).

Several bromate replacers are available, but ascorbic acid or vitamin C is by far the most popular and is permitted as a bread additive in most countries (Grosch, 1986). Ascorbic acid is a reducing agent, but functions as an oxidising agent in bread dough due to the presence of the ascorbic acid oxidase enzyme. This enzyme converts the ascorbic acid to dehydro-ascorbic acid in the presence of oxygen. Ascorbic acid can only function effectively as an oxidant in the presence of oxygen during mixing. L-threo-ascorbic acid is a stereoisomer of ascorbic acid that most strongly enhances strength, handling and baking properties of dough. Ascorbic acid strengthens the gluten; gas retention is thus improved and loaf volume increases (Kent & Evers, 1994). The effects of oxidising agents are usually observed as longer development times and greater stability (Hoseney, 1986). Ascorbic acid is used in breads and rolls at levels of 35-150 mg.kg<sup>-1</sup> to improve volume and texture.

#### 6.10.3 Emulsifiers

Emulsifiers are fatty substances that have both lipophilic and hydrophilic properties and belong in the general class of surface-active agents (surfactants). Emulsifiers reduce the surface tension between two normally immiscible phases enabling the liquids to form an emulsion (Dziezak, 1988; Flack, 1987; Krog, 1981). Emulsifiers function both as dough stabilisers, when emulsifiers interact with gluten proteins in dough, and as crumb softeners when the emulsifier complexes with the gelatinising starch during baking (Goesaert *et al.*, 2005). Glyceryl monostearate (GMS) has been used for many years in bread and bread rolls. It has powerful emulsifying properties and has the ability to complex with starch, slowing down the rate of staling (crumb firming). Lecithin is used for its properties of improving dough stability and tolerance, as well as loaf volume and texture; however, it is expensive, sticky and its use is declining. Lecithin is a compound of soy flour; therefore, soy flour can be used as an alternative.

Emulsifiers such as DATEM are commonly used in formulations to improve dough strength, produce dough that is drier and easier to process, exhibit better gas retention and result in bread

loafs with greater volume and finer texture (Hoseney, 1986). DATEM are one of the best dough stabilisers, but are usually the worst crumb softeners. Monoacylglycerols, on the other hand, have the best crumb softening effect, but they are inferior dough strengtheners (Stampfli & Nersten, 1995). Sodium and calcium stearoyl lactylates fatty acids (SSL/CSL) are produced from lactic acid with fatty acids. Used in bread (for their anti-staling properties), they can improve loaf volume as well as dough stability. Dough conditioners or dough improvers, usually contain a mixture of the following: emulsifiers such as DATEM and SSL, salts and acids such as calcium carbonate or monocalcium phosphate, reducing agents such as potassium bromate, ascorbic acid and potassium iodate, yeast foods such as ammonium salts and enzymes (Figoni, 2004). Calcium carbonate increases both water hardness and pH, whereas monocalcium phosphate increases water hardness while decreasing the pH. They are used for optimising gluten development by adjusting water hardness and pH.

#### 6.10.4 Chemical preservatives

Calcium propionate (CP) is an inhibitor of mould and rope (crumb deterioration due to *Bacillus subtilis*) (Brown, 1993). In South Africa, the cautious use of chemical preservatives like CP (CP legal limit 0.3% based on flour mass) in bread has largely reduced the problems of bread spoilage (Pattison, 2004). However, reports of cancer-like tumours in experiments where rats were fed with propionic acid at amounts of up to 4%, has led to the prohibition of CP usage in some European countries (Griem, 1985). Vinegar can be used as a substitute for CP; it has a better appeal on the label and can be listed as an ingredient and not as a preservative (Brown, 1993). The partial or complete replacement of CP with natural antimicrobials as inhibitors of rope (bacterial bread spoilage) and mould (fungal bread spoilers) in bread were investigated by Pattison *et al.* (2004). Combinations of acetic acid, lactic acid, a lactate-containing cocktail and calcium lactate were compared to CP in baking tests, with combinations of these natural antimicrobials successfully identified as potential replacements of CP.

#### 6.11 Bread-making processes

Bakery products are produced mainly according to three methods. The first method is the straight-dough method where mixing of ingredients are performed in one step (Mondal & Datta, 2008). The basic straight-dough bread-making method (AACC Approved Method 10-09; AACC, 2000) has a long fermentation time (180 min) whereas the optimised straight-dough bread-making method (AACC Approved method 10-10B; AACC, 2000) has a shorter fermentation time (90 min) (AACC, 2000). The sponge and dough or pound loaf method (AACC Approved Method 10-11; AACC, 2000) is the second method where mixing of ingredients is performed in two steps (Mondal &

Datta, 2008). The leavening agent is prepared during the first step. Yeast, water and flour are mixed together and left to develop for a few hours before it is mixed with the rest of the ingredients.

The Chorleywood Bread Process (CBP) is the third method where all the ingredients are mixed in an ultra-high mixer for a few minutes (Mondal & Datta, 2008); also described as a “no-time” dough-making process which uses mechanical development. Other “no-time” and mechanical dough development processes which remain in common use today includes, i.e. spiral mixing and mechanical dough development processes in Australia. The range of processes covered by spiral mixing are commonly seen in smaller bakeries and those in which the finest cell structure is not required, the mixing action is based on one or two spiral shaped beaters (Cauvain & Young, 2006). Currently mechanical dough development processes in Australia is similar to the CBP, but the tendency is to use higher work levels in the dough because of the stronger wheat varieties available in Australia and New Zealand (Cauvain & Young, 2006).

## *6.12 Stages during bread-making*

### *6.12.1 Mixing and kneading*

The major purpose of mixing is to blend the ingredients into a homogenous mixture, to develop the gluten matrix in wheat dough and to incorporate air. In under-mixed dough, starch and proteins are unevenly distributed and compact protein masses are stretched out into sheets during mixing (Moss, 1972). Over-mixing may cause damage to the gluten network (such as increased solubility of proteins and decreased extractability of lipids) (Evans *et al.*, 1981) and usually results in the formation of a sticky dough (Chen & Hoskeney, 1995). The reason for this is the mechanical forces applied to the dough decrease the molecular weight of the proteins. During kneading the dough's rheological properties are modified which improves its ability to expand when gas pressure increases due to the generation of carbon dioxide gas in the fermentation process (Cauvain & Young, 2006). Thus a visco-elastic network is formed that retains carbon dioxide (Autio & Laurikainen, 1997).

### *6.12.2 Dividing and moulding*

Dividing and moulding usually includes subdividing the bulk dough mass into unit pieces for subsequent processing (Cauvain & Young, 2006). For some products there is a preliminary modification of the shape of the divided dough pieces. There may be a short delay between the initial and the final shaping. During the final shaping of the dough pieces, the pieces obtain their required form. The gas cell structure is modified during dividing and moulding, causing small gas cells to burst and combine into larger ones (Autio & Laurikainen, 1997).

### 6.12.3 Fermentation (Proofing)

Fermentation occurs when yeast is present and the conditions are favourable (Cauvain & Young, 2006). The extent of bakers' yeast activity in bread dough depends on several factors such as the length of fermentation time, the dough temperature, the dough pH and the presence of inhibitors. Yeast fermentation generates carbon dioxide and the dough expands as a result of excess pressure in the gas cells (Bloksma, 1990). Gas cell stabilisation and gas retention are of considerable importance because they largely determine the crumb structure and volume of wheat bread (Brown, 1993). Gas retention refers to the ability of the dough to retain the carbon dioxide gas produced by yeast fermentation and to expand during proofing and baking (Cauvain & Young, 2006). If the dough is left to ferment in bulk after mixing, then the process is generally referred to as bulk fermentation (Cauvain & Young, 2006). Yeast fermentation increases with time and therefore any decrease in bulk fermentation time must be matched with an increase in yeast concentration to give the same gassing rate during final proofing. The rate of fermentation is also not only influenced by the dough temperature, but also by the temperature of the fermentation room. If the dough is cold, because the bakery is cold, the amount of yeast must be increased to ensure correct dough development and final proof times (Brown, 1993).

### 6.12.4 Baking

Baking is the last but most important step in the bread-making process (Mondal & Datta, 2008). Due to the combination of heat, moisture and time during baking, the starch granules gelatinise and swell. The gluten proteins undergo dramatic changes during baking; this is a combination of changes in protein surface hydrophobicity, sulphhydryl/disulfite interchanges and the formation of new disulphide cross-links (Goesaert *et al.*, 2005). The denaturation of proteins and the gelatinisation of starch, both affect the diffusion rate of water by releasing and absorbing water (Mondal & Datta, 2008). These two phenomena occur during the same temperature interval of 60-85°C and contribute to a change from dough to crumb.

Other major structural changes taking place during the heating of wheat dough are the expansion of the gas cells, melting of fat crystals and their incorporation on the surface of air cells and gas cells that rupture (Brooker, 1996). Heat and mass transfer are taking place simultaneously during bread baking utilising the four phases in the transport process, i.e. solid, liquid water, vapour and CO<sub>2</sub> (Mandal & Datta, 2008). The rising temperature during baking causes the thermal expansion of vapour and raises the saturation pressure of water within the dough. Carbon dioxide also plays an important role in the expansion of bubbles during bread baking. It is released from the bread when the bubble wall starts to break under pressure, making the porous structure more continuous and open.



The crust acts as a barrier towards weight loss during baking (Mondal & Datta, 2008) and forms where the maximum evaporation takes place (Therdthai *et al.*, 2002). Crust formation is one of the limiting factors restricting the expansion of the dough during baking (Zhang *et al.*, 2007). The formation of crust and browning during baking appear to be the primary contributors to the formation of bread flavour. The browning is mainly a result of the Maillard reaction and occurs when temperatures are greater than 110°C. The Maillard reaction is important for the formation of colour and aroma in the bread crust, but may also be associated with the formation of toxic compounds such as acrylamide which is carcinogenic (Zanoni, 1995).

## 7. Bread staling

Bread staling has been studied for nearly 150 years, but the precise mechanism is still far from understood (Gray & Bemiller, 2003). Bechtel *et al.* (1953) defines staling as a term which indicates decreasing consumer acceptance of bakery products caused by changes in the crumb, other than those resulting from the action of spoilage organisms. Bread staling falls into two categories, i.e. **crust staling** and **crumb staling**. **Crust staling** is generally caused by moisture transfer from the crumb to the crust (Lin & Lineback, 1990), resulting in a soft, leathery texture and is generally less objectionable than crumb staling (Newbold, 1976). **Crumb staling** is more complex, more important, but less understood (Gray & Bemiller, 2003). The firmness of bread varies with position within a loaf, the maximum firmness occurring in the central portion of the crumb.

Retrogradation of starch remains the most widely accepted factor contributing to bread staling, but there is also good evidence that there is no cause-and-effect relationship between retrogradation and staling (Gray & Bemiller, 2003). While amylopectin retrogradation is believed to play the major role, amylose is also thought to be involved. Moisture content and transfer of moisture between bread components is believed to be a significant factor contributing to bread staling. Moisture transfer from gluten to starch might be involved in the staling process. Kim and D'Appolonia (1977a) found pentosans had a definite effect on retarding starch retrogradation in wheat starch gels, with the effect of water-insoluble pentosans being more pronounced. They reported water-soluble pentosans reduced retrogradation by acting on amylopectin and water-insoluble pentosans reduced the degree of retrogradation of both amylase and amylopectin. Kim and D'Appolonia (1977b) found similar results when they studied the effect of pentosans on staling in a bread system. The results indicated the basic mechanism of bread staling was unchanged; thus, it was suggested pentosans decreased the staling rate by reducing the amounts of starch components available for retrogradation.

Changes in bread crumb characteristics such as taste and aroma, increased hardness, increased opacity, increased crumbliness, increased starch crystallinity, decreased absorptive capacity,



decreased susceptibility to  $\alpha$ -amylase and decreased soluble starch content have been used as basis to determine the degree of staling (Geddes & Bice, 1946). However, the measurement of the increase in crumb firmness remains the most widely used indicator of staling (Gray & Bemiller, 2003). As bread stales, the texture of the crumb changes from a relatively soft, spongy texture to a texture that is firm and crumbly. Therefore, numerous compressibility methods have been developed to quantify the firming of the bread, which have been shown to correlate with bread staling as measured by consumer acceptability. Most methods measure the force applied by compressing a sample a specific distance. AACC Approved Method 74-09 (AACC, 2000) uses the Instron Universal Testing Machine to determine the degree of firmness in white pan bread crumb. A 25% compression depth (as specified in the AACC Approved Method 74-09) was confirmed to be the most effective method for detecting significant differences in bread firmness due to staling (Baker *et al.*, 1988). Other methods used for measuring the degree of staling include, thermal analysis, near NIR spectroscopy, nuclear magnetic resonance spectroscopy, X-ray crystallography, microscopy and sensory/organoleptic tests (Gray & Bemiller, 2003).

## 8. Baking enzymes

Enzymes are proteins that are produced by all living organisms (Law, 2002). They speed up chemical reactions selectively as part of essential life processes such as digestion, respiration, metabolism and tissue maintenance. Enzymes are in other words highly specific biological catalysts and work under more or less mild conditions of temperature and pH. Most enzymes only catalyse reactions between one type of chemical compound (its substrate) and this defines its specificity and provides the basis of its classification and name. Enzymes have trivial names derived from the substrate name with ‘-ase’ added and systematic names or an International Union of Biochemistry Enzyme Commission (IUB or EC) number. Most commercial enzyme preparations contain not only the specific enzyme whose activity is printed on the label, but also other enzymes that happened to be produced by the same source material or organism. In food enzyme legislation, most enzymes are regarded as processing aids because they are added during processing for technical reasons. In the USA food enzymes are regulated by the Food and Drug Administration (FDA) and rely heavily on the Generally Recognised as Safe (GRAS) status. Food enzymes are also regulated in the United Kingdom (UK) and European Union (EU) member states. For an enzyme to be regarded as safe for human consumption, it must pass stringent testing to prove absence of toxins, allergens, heavy metals, pathogenic microorganisms and other hazardous contaminants, as specified by the WHO/FAO Joint Expert Committee for Food Additives (JECFA) (Law, 2002). The classification, function, specificity and optimum conditions of the three major classes of enzymes used in this study will be discussed further.

## 8.1 Amylases

### 8.1.1 Classification

Glycoside hydrolases (GH) (EC 3.2.1.-) are a widespread group of enzymes which hydrolyse the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Anon., 2001). Starch degrading enzymes can be classified into three main groups based on their mode of action, i.e. endo-amylases, exo-amylases and debranching enzymes (Muralikrishna & Nirmala, 2005).

### 8.1.2 Function and specificity

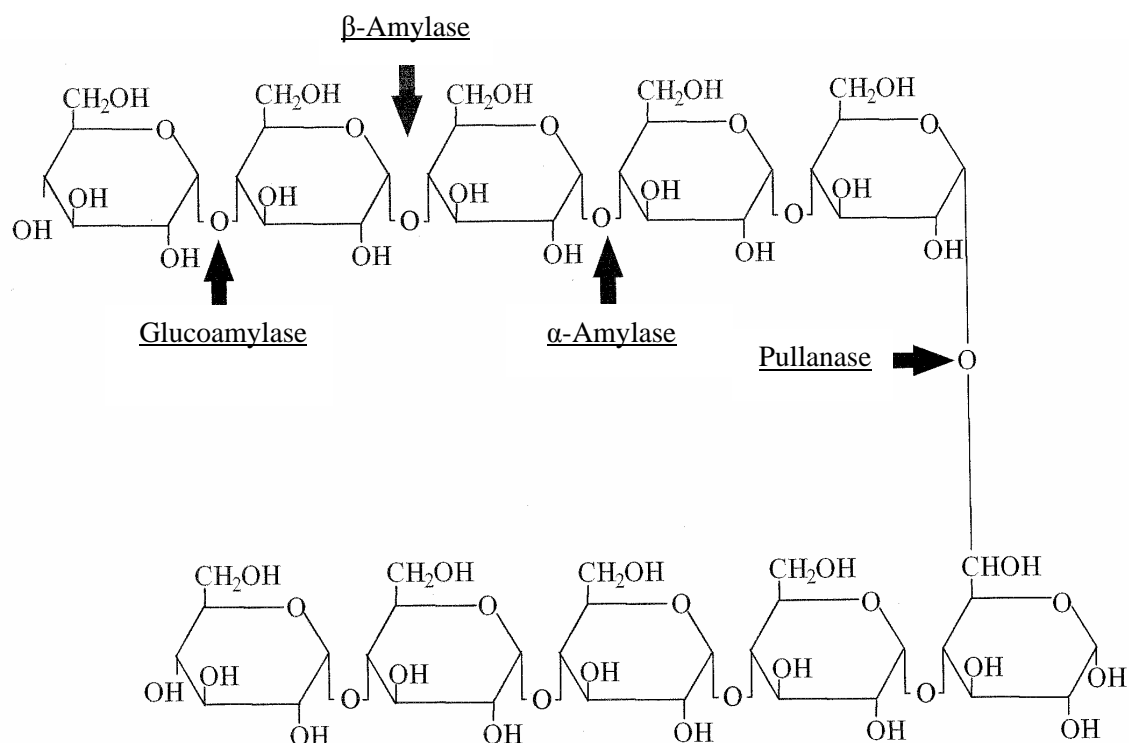
Endo-amylases, also known as  $\alpha$ -amylases (EC 3.2.1.1; GH 13), randomly hydrolyse the  $\alpha$ -(1,4)-glycosidic linkages of starch (**Fig. 2.4**) (Hill & MacGregor, 1988). The products of hydrolysis are oligosaccharides with varying chain lengths, containing the  $\alpha$ -configuration at the C1 of the reducing glucose unit. Endo-amylases hydrolyse the bonds located in the inner regions of the starch molecule, yielding low molecular weight  $\alpha$ -dextrins (Bowles, 1996; Hoseneey, 1994).

$\beta$ -amylases (EC 3.2.1.2; GH 14) and glucoamylases (amyloglucosidase) (EC 3.2.1.3; GH 15) are exo-amylases which hydrolyse the  $\alpha$ -(1,4)-linkages at the non-reducing ends of starch molecules through successive removal of maltose or glucose respectively, in a stepwise manner (**Fig. 2.4**) (Banks & Greenwood, 1975). The products of hydrolysis have the  $\beta$ -configuration at the C1 of the reducing glucose unit due to inversion of configuration of the end-product.  $\beta$ -amylases potentially degrade starch to  $\beta$ -maltose and  $\beta$ -limited dextrins (Bowles, 1996; Hoseneey, 1994). The branch points, containing  $\alpha$ -(1,6) glycosidic linkages, are resistant to attack by  $\alpha$ - and  $\beta$ -amylases and result in  $\alpha$ - or  $\beta$ -limit dextrins, respectively. Debranching enzymes like pullulanases (E.C 3.2.1.41) and isoamylases (E.C. 3.2.1.68) can hydrolyse these  $\alpha$ -(1,6) bonds, resulting in the removal of the side chains (Goesaert *et al.*, 2005).

The  $\beta$ -amylases are almost always in abundance in flour, but the  $\alpha$ -amylase activity is low. Therefore,  $\alpha$ -amylases are added to flour to optimise the  $\alpha$ -amylase activity (Drapron & Godon, 1987). The supplemented  $\alpha$ -amylases facilitate maltose production in combination with the endogenous  $\beta$ -amylases by degrading the damaged starch molecules during the dough stage and generating low molecular weight dextrins (Linko *et al.*, 1997). When the  $\alpha$ -amylase activity in dough is insufficient, the maltose content remains too low, resulting in the dough raising poorly (Linko *et al.*, 1997). This problem can be alleviated by addition of 0.3% of malt flour or fungal  $\alpha$ -amylases (at the mill or at the bakery). Amylases produce an increase in fermentable sugars and reducing sugars. The maltose released can then be used by the yeast as a fermentable sugar. The

increased levels of reducing sugars promote the generation of Maillard reaction products, which intensify bread flavour and crust colour (Bowles, 1996; Drapron & Godon, 1987).

Fungal amylases have low thermal stability and most of their activity is lost during starch gelatinisation. Some bacterial  $\alpha$ -amylases, however, are thermo stable and may survive the baking process (Bowles, 1996).



**Figure 2.4** Schematic representation of starch and the hydrolytic enzymes required for its degradation. The black arrows indicate the sites of attack of the various enzymes (Rose, 1998).

Treatment with amylase lowers the viscosity of the dough, improves handling and results in a softer and larger loaf of bread. During the baking process, starch gelatinises and proteins denature, which forms a rigid structure releasing water to the gelatinised starch.

When the  $\alpha$ -amylase activity of flour is high (i.e. falling number is low), excessive starch breakdown occurs during the early stages of oven baking, resulting in a moist, sticky and rubbery bread crumb with a reduced volume (Linko *et al.*, 1997). This problem cannot be rectified by mixing the wheat with higher quality wheats. The traditional method of reducing  $\alpha$ -amylase activity, especially with rye breads, has been sourdough baking with lactic acid bacteria (Linko *et al.*, 1997). When the pH of the dough drops below 4.5, the  $\alpha$ -amylases are inactivated. The controlled addition of an amylase of intermediate thermostability can be used to reduce starch retrogradation and to approximately double the shelf-life of bread (Linko *et al.*, 1997). The use of

$\alpha$ -amylases to retard bread staling can be enhanced by the addition of pullulanases from *Bacillus acidopullulyticus*.

### 8.1.3 Optimum conditions

The pH optima values of cereal amylases are in the range of 4.5-5.5. Temperature is one of the most important parameters that affect the rate of enzyme hydrolysis. The temperature optima of known cereals amylases are between 40 and 55°C. Above this temperature range, most of the cereal amylases undergo inactivation. Generally,  $\alpha$ -amylases are more thermostable compared to  $\beta$ -amylases (Nirmala & Muralikrishna, 2003). Commercial  $\alpha$ -amylases differ in temperature optima; in general, fungal enzymes have a lower (40-50°C) temperature optima and bacterial enzymes have a higher (70-80°C) temperature optima (Hamer, 1991).

## 8.2 Xylanases

### 8.2.1 Classification

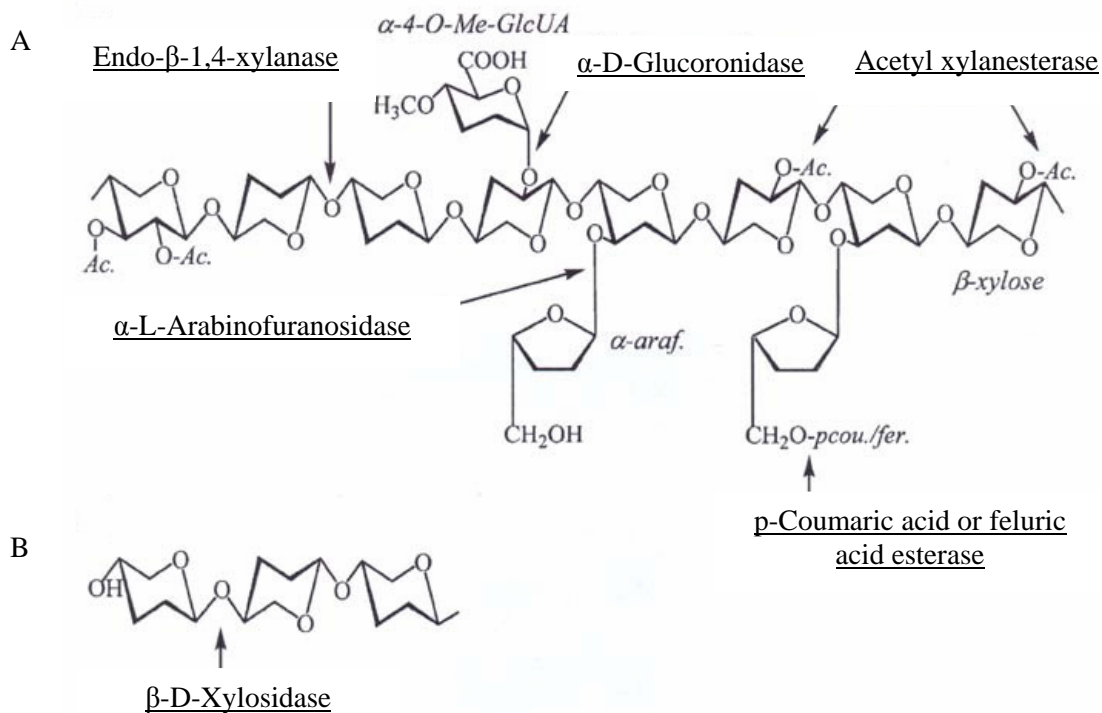
Based on amino acid sequence and structural similarities, endoxylanases have been classified into GH families 5, 7, 8, 10, 11, 16, 26, 43, 52 and 62 (Coutinho & Henrissat, 1999). However, according to Collins *et al.* (2005) only the sequences classified in GH families 5, 7, 8, 10, 11 and 43 contain truly distinct catalytic domains with a demonstrated endoxylanase activity. Only xylanases from the GH families 10 and 11 has as yet found application in the baking industry. The potential of family 8 xylanases as technological aids in bread-making was, however demonstrated in a study by Collins *et al.* (2006). Enzymes belonging to families 10 and 11 differ in molecular weight, optimum temperatures, isoelectric points and substrate specificity (Hille & Schooneveld-Bergmans, 2004).

### 8.2.2 Function and specificity

Endogenous xylanases hydrolyse the endosperm cell wall upon germination making the storage components, such as starch and gluten proteins, available to amylases and proteases (Courtin & Delcour, 2002). The level of xylanase activity in wheat flour is usually too low to deliver optimum effect in bread-making (Hille & Schooneveld-Bergmans, 2004). Therefore, the most important type of hemicellulase used in bread-making is microbial derived endoxylanases (EC 3.2.1.8) (Goesaert *et al.*, 2005).

The complete degradation of AX requires cooperative action of both depolymerising enzymes, which hydrolyse the xylan backbone, and side-group cleaving enzymes, which remove the side-groups from the xylan backbone (Soerensen *et al.*, 2003; Collins *et al.*, 2005). Enzymes that remove side chains from the xylan backbone make the latter more accessible for depolymerising enzymes which often prefer less substituted regions. Endo- $\beta$ -D-1,4-xylanases (endoxylanases) are the most

important AX depolymerising enzymes as they cleave internal  $\beta$ -1,4-xylosidic linkages in the xylan backbone (**Fig. 2.5**).



**Figure 2.5** Hydrolysis of AX by different xylanolytic enzymes (A) and hydrolysis of xylooligosaccharide by  $\beta$ -D-xylosidase (B) (Collins *et al.*, 2005).

The impact of endoxylanases on AX functionality in bread-making strongly depends on their selectivity towards WE-AX and WU-AX substrates. Optimal levels of endoxylanase activity with specificity towards WU-AX will result in a decrease in the level of WU-AX and an increase in the level of solubilised AX, which has a positive effect on dough and bread properties (Courtin *et al.*, 2002). These enzymes catalyse the conversion of WU-AX to HMW AX during which the WU-AX is partially broken down and water is released. This brings the HMW AX in solution and results in an increase in viscosity. According to Courtin *et al.* (1999; 2001) the use of suitable endoxylanases increases dough stability. This means that during fermentation, the dough retains its optimal volume for a longer period of time and is thus more resistant to mechanical stress. It further implies that during the initial stage of baking the oven rise is significantly prolonged, leading to a higher loaf volume and finer, softer and more homogeneous bread crumb.

Water released during the partial degradation of WU-AX moves to the gluten phase. This leads to an increase in volume, extensibility and gas retention of the dough resulting in better oven spring. The starch granules absorb water and expand, leading to amylose and amylopectin leaching out and forming a gel during baking (Courtin & Delcour, 2002). The endoxylanases also remove the

insoluble AX that interferes with the formation of the gluten network and thus increases the stability of the dough system due to increased viscosity. It yields a more stable and flexible dough that is easier to handle and improves oven spring, loaf volume and crumb structure (Courtin & Delcour, 2002). In contrast to the above, endoxylanases that hydrolyse WE-AX or solubilise AX will reduce their molecular weight and result in little, if any, improvement (Goesaert *et al.*, 2005). In both cases, excessive levels of endoxylanase activity results in slack and sticky doughs and loaves with poor crumb structure and colour, gas cell distribution and crust colour (Goesaert *et al.*, 2005).

### 8.2.3 Optimum conditions

In general, fungal endoxylanases are more active under acidic conditions and are stable over a broad pH range (pH 3.0-10.0), while bacterial endoxylanases have a higher pH optimum (pH 3.5-5.5) and narrow pH range (pH 5.0-7.3). Bacterial and fungal endoxylanases typically have temperature optima between 40 and 50°C (Dekker & Richards, 1976). Plant endoxylanases are generally most active at pH 5.0 and 40°C (Dornez, 2007).

### 8.2.4 Endoxylanase inhibitors

The plant cell wall is a resilient and structurally heterogeneous barrier composed of complex polysaccharides and diverse proteins (O'Neil & York, 2003). In addition to providing structural support, cell walls also act as an important line of defence against pathogens. The main components of primary cell walls are members of two polysaccharide networks; one consisting of cellulose and hemicellulose, and the other consisting of pectic polysaccharides (Juge, 2006). To penetrate and use plant cell walls nutritionally, pathogens secrete a consortium of polysaccharide degrading enzymes, including exo- and endopolygalacturonases, pectin lyases, pectate lyases, acetyl esterases, pectin methylesterases, xylanases and a variety of cellulases (Lebeda *et al.*, 2001).

As a defence mechanism, plants resist hydrolytic attack by deploying inhibitor proteins to a range of enzymes including cell wall degrading enzymes. Different inhibitors have been identified in wheat, i.e. *Triticum aestivum* endoxylanase inhibitor (TAXI) (Debyser *et al.*, 1999), endoxylanase inhibiting protein (XIP) (Mc Lauchlan *et al.*, 1999) and the thaumatin-like xylanase inhibitor, (TL-XI) (Fierens *et al.*, 2007). TAXI inhibits family 11 endoxylanases (Gebruers *et al.*, 2001), XIP inhibits both family 10 and 11 fungal endoxylanases (Flatman *et al.*, 2002) and TL-XI inhibits bacterial and fungal endoxylanases, but not plant endoxylanases. Inhibitor concentrations in wheat flour are in the range of 15 mg.kg<sup>-1</sup> or higher for both TAXI and XIP and can vary two to three fold depending on the variety (Gebruers *et al.*, 2002). Sensitivity towards these endoxylanase inhibitors is a factor that strongly influences endoxylanase functionality in bread-making. Debyser *et al.* (1999) observed the inhibition of endoxylanases derived from *Aspergillus niger* and *Bacillus*

*subtilis* by TAXI, whereas endoxylanases from *Aspergillus aculeatus* and the endoxylanases from a rumen microorganism were not inhibited by TAXI. Endoxylanase derived from *A. niger* have previously been used in baking tests. It was observed the endogenous wheat flour endoxylanases have a considerably positive effect on bread volume and that they were inhibited by TAXI (addition of 25 mg of purified TAXI to the dough). Most microbial endoxylanases are susceptible to inhibition by endoxylanase inhibitors, present in large quantities in wheat. Only bacterial GH family 10 endoxylanases and some individual endoxylanases, such as endoxylanases derived from *A. aculeatus*, are insensitive to inhibition.

So far, all endoxylanases in wheat whole meal and flour were considered to be endogenous. However, the addition of TAXI-type inhibitors to wheat flour decreases bread loaf volume, implying the presence of microbial endoxylanases in the flour (Debyser *et al.*, 1999). Addition of TAXI-type inhibitors reduces the endoxylanase activity in wheat flour extracts (Gys *et al.*, 2004). TAXI-type inhibitors are not able to inhibit plant endoxylanases, thus suggesting the contamination of flour with wheat kernel associated microbial endoxylanases. According to Debyser *et al.* (1999) breeding TAXI-deficient wheat cultivars or cultivars with low levels of expression of this inhibitor may be important for improving bread-making performance.

### 8.3 Cellulases

#### 8.3.1 Classification

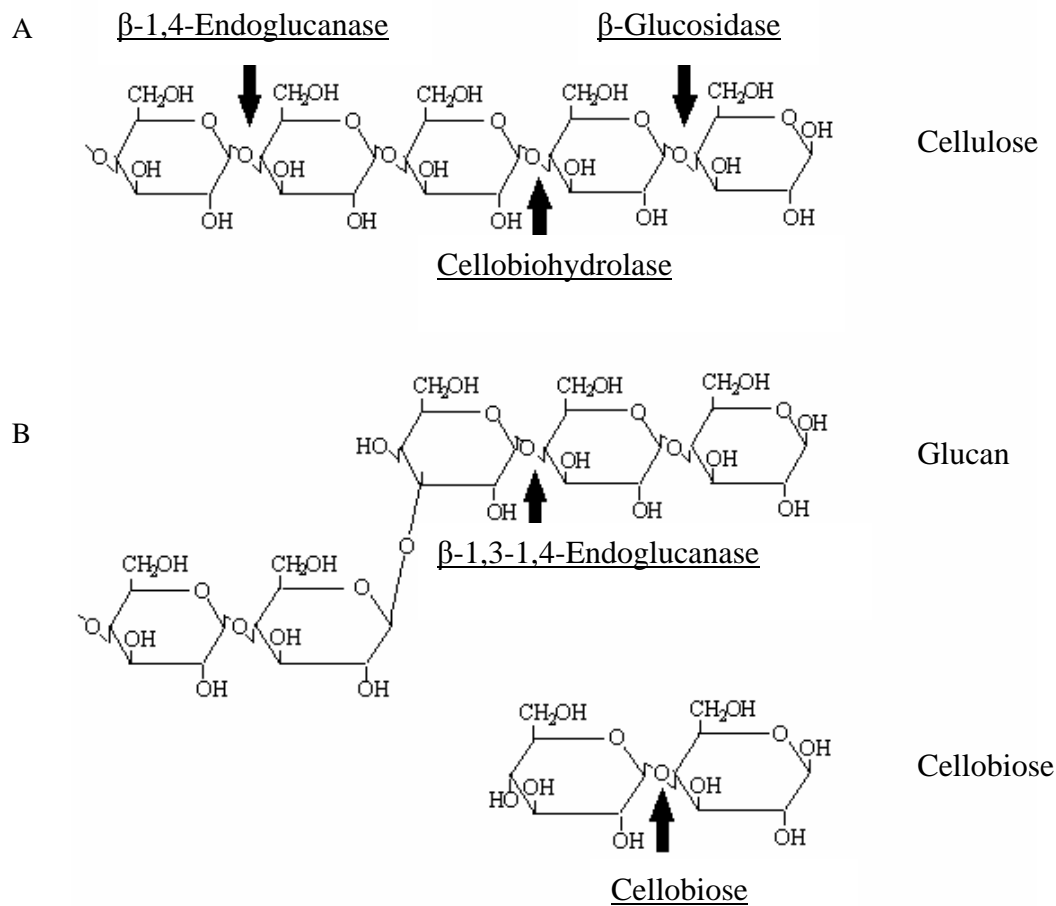
The term cellulase (E.C. 3.2.1.4) refers to a collection of different enzymes (produced from fungi, bacteria or protozoans) that work together to degrade cellulose. These enzymes include endoglucanases ( $\beta$ -1,4-glucanase or endo-1,4- $\beta$ -D-glucanase) which is responsible for the endohydrolysis of 1,4- $\beta$ -D-glucosidic linkages in cellulose, lichenin and cereal  $\beta$ -glucans (Anon., 2001).

#### 8.3.2 Function and specificity

Cellulose is predominantly present in the cell walls of all plants, representing the largest form of fixed carbon in nature (Beguin & Aubert, 1994). Cellulose is one of the smaller constituents of non-starch polysaccharides and is made up of long chains of (1,4)- $\beta$ -linked-D-glucose units (Hille, 2005). The hydrolysis of cellulose is very difficult due to its insolubility and dense crystalline structure. The conversion of native cellulose requires the synergistic action of at least three different enzymes, a  $\beta$ -1,4-endoglucanase ( $\beta$ -1,4-glucan glucanohydrolase EC 3.2.1.4), a  $\beta$ -1,4 exoglucanase (cellobiohydrolase EC 3.2.1.91 and exo- $\beta$ -1,4-glucohydrolase EC 3.2.1.74) and a  $\beta$ -glucosidase ( $\beta$ -D-glucoside glucohydrolase EC 3.2.1.21) (**Fig. 2.6**) (Aristidou & Penttilä, 2000).



Parallel cellulose chains form fibrils and are partly intertwined with AX. The cellobiohydrolases play a specific role in degrading these crystalline structures (Hille, 2005). The cellulose binding domain (also known as the cellulose binding module) binds the cellobiohydrolase to the fibril, forcing the cellulose strands apart, after which the active site of the cellobiohydrolase is able to split off cellobiose units from the cellulose chain. The endoglucanases act randomly to hydrolyse the amorphous and soluble cellulose derivatives with the release of oligosaccharides. These enzymes can also result in the endohydrolysis of 1,4- $\beta$ -D-glucosidic linkages in lichenin and cereal  $\beta$ -glucans (Anon., 2001). The  $\beta$ -glucosidases complete the hydrolysis by removing glucose residues from the non-reducing end of oligosaccharides (Aristidou & Penttilä, 2000). Cellobiases are part of the  $\beta$ -glucosidase family, but only attack cellobiose resulting in the production of glucose as an end-product.



**Figure 2.6** A schematic representation of a hypothetical (A) cellulose and (B) glucan chain and the various sites of attack by the enzymes involved in the degradation (Rose, 2003).



The hydrolysis of cellulose enables endoxylanase to hydrolyse arabinoxylans which are partly intertwined with the cellulose. Endoglucanases, cellobiohydrolases and  $\beta$ -glucosidase have been shown to work synergistically (Hille, 2005). The use of commercial cellulases as an additive in different bread-making processes at optimum levels, resulted in an increase in bread loaf volumes, bread scores and reduced crumb firmness (Harada *et al.*, 2000; 2005).

### 8.3.3 Optimum conditions

In general, fungal cellulases have a temperature optima between 50 and 80°C and their optima pH range is between 5.0 and 7.0. Bacterial cellulases have a similar temperature optimum, but a higher optimum pH range between 6.0 and 9.0 (Baldrain & Valaskova, 2008).

## 9. The role of enzymes in bread-making and staling

Cereal grains contain a large number of specific enzymes. The variation in their activity levels affects the quality of cereal raw materials. This variation is due to differences in wheat cultivars used and the climatic conditions during growing and harvesting.

Commercial enzyme mixtures consist primarily of the same type of enzymes, but are usually from microbial origin (Poutanen, 1997). Amylases and proteases have long been used in the baking industry. The addition of amylases mainly aims at optimising the amylase activity of the flour and retard bread staling (Geosaert *et al.*, 2005). There are two sources of supplementation, enzyme active malt flour (provides cereal amylase) or commercial  $\alpha$ -amylase (fungal or bacterial) (Brown, 1993). The development and commercialisation of fungal and bacterial  $\alpha$ -amylase occurred in the late 19<sup>th</sup> and early 20<sup>th</sup> centuries, respectively (Muralikrishna & Nirmala, 2005). By the 1930s these enzymes were used commercially in a variety of applications. The effectiveness of bacterial  $\alpha$ -amylases as an anti-staling agent in baked foods was realised in the middle of the 20<sup>th</sup> century (Miller *et al.*, 1953). The development of genetic engineering tools in 1970s successfully paved the way for production of cloned amylases in 1980 and 1990s for industrial applications (Brumm *et al.*, 1991; Zemen & McCrea, 1985). It has been reported that the rates and degrees of firming in baked goods can be reduced and the texture, flavour, aroma and general qualities can be improved with the use of  $\alpha$ -amylases. Fungal, cereal and bacterial  $\alpha$ -amylases all appeared to improve softness retention of bread to an extent related to their heat stability (Miller *et al.*, 1953).

Enzyme supplements, either amylases or proteases are most commonly used in commercial baking to reduce the rate of bread staling (Bowles, 1996). Proteases are usually added to bread to improve flavour profiles, flow characteristics, machining properties, gas retention and mixing time. The reducing agent sodium bisulphite completely or partially destroys vitamin B1 (thiamine) present in flour, leaving proteases as an attractive alternative (Anon., 2005). In a study by Caballero

*et al.* (2006) it was shown that the addition of amylase, xylanase and protease exhibited a significant anti-staling effect according to crumb texture evolution.

Enzyme hydrolysis of non-starch polysaccharides can lead to the improvement of rheological properties of dough, bread specific volume and crumb firmness (Martinez-Anaya & Jimenez, 1997). At the optimum dosage, xylanases can improve dough machinability, dough stability, oven spring, loaf volume, crumb structure and shelf-life (Hamer, 1995; Poutanen, 1997). Endoxylanase of fungal and bacterial origin perform equally well in improving bread characteristics such as loaf volume, appearance, opening of the cut and crumb texture, whereas the bacterial endoxylanases also introduces initial crumb softness (Hille, 2005). Courtin and Delcour (2002) showed the addition of enzymes that act on the WU-AX both improved the loaf volume and overall bread-making score. Crumb structure was also shown to be positively affected (Rouau *et al.*, 1994). A similar trend was observed for purified endoxylanase. Popper (1997) described marked increases in both oven rise and final loaf volume when selected endoxylanases were added to a bread recipe. These were further accompanied by a fine, soft crumb and increased shelf-life. Xylanase of *A. niger var awamori* improved the quality of bread by increasing the specific volume. This was further enhanced when amylases in combination with the xylanases were used (Maat *et al.*, 1992). Work carried out by Hille & Schooneveld-Bergmans (2004) showed endoxylanases of both fungal and bacterial origin can improve bread quality in terms of loaf size and shape, as well as crumb texture and softness. Hille (2005) showed cellulases and endoxylanases work synergistically in improving bread quality such as loaf volume and crumb softness.

Glucose oxidase (EC 1.1.3.4) is the currently preferred enzyme alternative to chemical oxidising agents for bread improvement (Bonet *et al.*, 2006). The hydrogen peroxide produced during the glucose oxidase reaction promotes the formation of disulphide linkages in gluten proteins and the gelation of water soluble pentosans (Hoseney & Faubion, 1981).

Lipolytic enzymes hydrolyse the lipid structure present in wheat flour, produce *in situ* emulsifying agents and can be used to replace chemical emulsifiers such as DATEM, SSL and GMS (Hille, 2005). Lipases increase dough strength and stability, thus improving dough machinability. They also increase volume and crumb softness and can result in an improved, more uniform crumb structure (Oleson *et al.*, 1994). Oleson (1994) also reported lipases may have anti-staling properties. The enzyme lipoxygenase can be used as flour whiteners instead of chemical bleaching agents (Brown, 1993). Lipoxygenases have been reported to have a crumb softening effect when active in bread. Enzyme-active soy flour is a major source of lipoxygenases (Van Ejik & Hille, 1996).

## 10. Conclusion

Wheat flour consists of starch, water, proteins and minor constituents, such as non-starch polysaccharides and lipids. Different chemical analyses as well as rheological tests are routinely performed on wheat flour to ensure a consistent end-product. The basic ingredients added during wheat flour bread-making are flour, water, yeast, salt, sugar and shortening. Apart from these ingredients soyflour, dried gluten, malt flour or fungal  $\alpha$ -amylases and additives can also be added to improve the product quality.

Wheat flour also contains several enzymes but they are not always present in quantities adequate for bread baking. Through the process of molecular genetics, enzymes can be produced (Hille, 2005) and can be used instead of chemical additives or to add new functionalities to a product (Linko *et al.*, 1997). These commercial enzyme mixtures consist primarily of the same types of enzymes as those that are endogenously present in wheat, but are usually from microbial origin (Poutanen, 1997). These microbial enzymes are safe as they are easily inactivated by the baking temperatures (Hamer, 1995). Functionalities includes improving dough strength and stability, increasing gas retention, improving loaf volumes, improving crumb structure and softness and increasing shelf-life.

There are three different bread-making processes which are used for the production of bakery products and four stages during bread-making. Each of these stages has a specific role in bread-making and influences the end-product quality. After the bread is baked it begins to lose its freshness and stales. Bread staling can be divided into crust staling and crumb staling and the measurement of the increase in crumb firmness remains the most widely used indicator of staling (Gray & Bemiller, 2003). Three important enzymes and their role in bread-making and staling are discussed.

The use of enzymes in new products will continuously be evaluated as consumers are always looking for new and improved products and products which offer more convenience due to an extended shelf-life. It is further expected that the market for baking enzymes will continue to grow. In developed markets such as the USA and the EU the growth is mostly generated in new segments such as frozen dough and pre-baked bread or by using new enzymes such as lipase. In emerging markets the demand is primarily for baking enzymes, such as amylases and xylanases (Popper & Kutschinski, 2007).

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## **CHAPTER 3**

**The effect of commercial and pure endoxylanases, endoglucanases and their combination on wheat flour bread quality tested on small-scale**

## The effect of commercial and pure endoxylanases, endoglucanases and their combination on wheat flour bread quality tested on small-scale

### Abstract

Commercial and pure endoxylanases and endoglucanases were evaluated for their ability to improve wheat flour bread quality. A commercial endoxylanase from *Aspergillus niger* and a commercial endoglucanase from *Trichoderma reesei* were evaluated together with a pure endoxylanase (GH family 11) and pure endoglucanase (GH family 7) from *Trichoderma* sp. The effects of these enzymes on dough quality, bread weight, bread height and softness of crumb were analysed. A digital imaging system (C Cell) was used to evaluate bread slice characteristics and overall crumb texture. Compared to the control both pure and commercial endoxylanases significantly ( $P < 0.05$ ) improved bread height and softness of crumb and the pure endoxylanase also increased slice brightness. Both the pure and commercial endoglucanases significantly ( $P < 0.05$ ) increased softness of the crumb and slice brightness. Different enzyme combinations were also tested with three combinations resulting in a significant ( $P < 0.05$ ) increase in bread height. All three combinations of the respective pure endoxylanase and endoglucanase showed tendencies to decrease the number of holes, area of holes and the volume of the holes, which may lead to a finer crumb. These results were, however, not significant ( $P > 0.05$ ). The enzyme combinations did not significantly ( $P > 0.05$ ) increase slice brightness or crumb softness.

### Introduction

Wheat flour generally contains approximately 2-3% arabinoxylans whereas wholemeal wheat flour can contain up to 5% arabinoxylans (Baillet *et al.*, 2003). Arabinoxylans (AX) can be divided into water-extractable and water un-extractable fractions. The water un-extractable arabinoxylans (WU-AX) makes up 70-75% of AX (Courtin & Delcour, 2002) and has been reported to be able to hold ten times their weight in water (Kim & D'Appolonia, 1977). Microbial derived endoxylanases (E.C. 3.2.1.8) are able to hydrolyse the arabinoxylan backbone internally and are usually classified into glycoside hydrolase families 10 and 11 (Goesaert *et al.*, 2005). The use of endoxylanases which attack the WU-AX cause a reduction in the level of AX and an increase in the level of soluble AX. This has a positive impact on dough and bread properties (Goesaert *et al.*, 2005). The use of suitable endoxylanases increases dough stability during fermentation; the dough retains its optimal volume for a longer period of time and is more resistant to mechanical stress. It also implies that during the initial stages of baking the oven rise is prolonged leading to a higher loaf volume and a finer, softer and more homogenous crumb (Courtin *et al.*, 1999; 2001).

Enzyme hydrolysis of non-starch polysaccharides can lead to the improvement of rheological properties of dough, bread specific volume and crumb firmness (Martinez-Anaya & Jimenez, 1997). At the optimum dosage, xylanases can improve dough machinability, dough stability, oven spring, loaf volume, crumb structure and shelf life (Hammer, 1995; Poutanen, 1997). Popper (1997) described marked increases in both oven rise and final loaf volume when selected endoxylanases were added to a bread recipe. These were further accompanied by a fine, soft crumb and increased shelf life. It has been shown both fungal and bacterial endoxylanases are able to improve bread quality in terms of loaf size and shape as well as texture and crumb softness (Hille, 2005). The use of endoxylanases derived from *Aspergillus foetidus* in whole wheat bread improved the volume, specific volume and decreased the crumb firmness (Shah *et al.*, 2005). In a study by Caballero *et al.* (2006) it was shown that amylases and endoxylanases exhibited a significant anti-staling effect. When endoxylanases derived from *Thermotoga maritima* was used, it led to improvements in the specific volume and crumb structure and had an anti-staling effect (Jiang *et al.*, 2004). When fungal endoxylanases was used it produced a slightly sticky dough, whereas the addition of bacterial endoxylanases resulted in a dry, elastic, tight dough (Hille & Schooneveld-Begmasn, 2004).

Cellulose is one of the smaller constituents of non-starch polysaccharides and because of its fibril structure it is not easily degraded (Hille, 2005). Cellobiohydrolases, endoglucanases and  $\beta$ -glucosidases work together to break down the cellulose polymer (Aristidou & Penttilä, 2000). Cellulases and endoxylanases have been shown to work synergistically as the hydrolysis of cellulose enables endoxylanase to hydrolyse arabinoxylans, which are partly intertwined with the cellulose (Hille, 2005). The effect of endoglucanases on bread making is less studied than that of endoxylanases. The use of commercial cellulases as an additive in different bread making processes resulted in an increase in bread loaf volumes, bread scores and reduced crumb firmness (Harada *et al.*, 2000; 2005). When combining fungal and bacterial endoxylanases with cellulases, smaller quantities of each enzyme are required and can result in an increase in softness over time (Hille, 2005).

In the current study the effect of commercial endoxylanases and endoglucanases, which is known to contain small amounts of other enzymes such as  $\alpha$ -amylases and cellobiohydrolases, was evaluated. In addition the effect of these enzymes was also evaluated in a pure form consisting of only endoxylanase and endoglucanase, respectively.

The specific objectives of this study were therefore to:

- determine the activity of the respective commercial and pure endoxylanases and endoglucanases against different substrates;



- determine the optimum concentration of each enzyme by means of small-scale (100 g) baking trials;
- evaluate the effect of the enzymes on dough and bread quality characteristics, i.e. loaf weight, loaf height, crumb texture, slice brightness and crumb softness; and
- evaluate the effect of a combination of the respective commercial and pure endoxylanases and endoglucanases on dough and bread quality characteristics, i.e. loaf weight, loaf height, crumb texture, slice brightness and crumb softness.

## Materials and methods

### *Flour, enzymes, ingredients and chemicals*

Commercial white bread flour supplied by Pioneer Foods trading as Sasko (Paarl, South Africa) was used. The commercial enzymes used included  $\alpha$ -amylase (*Aspergillus oryzae*), endoxylanase (*A. niger*) and endoglucanase (*Trichoderma reesei*). The pure enzymes used was endoxylanase (*T. reesei* Xyn2, heterologously expressed in *A. niger* D15) (GH family 11) and endoglucanase (*T. reesei* egI, heterologously expressed in *A. niger* D15) (GH family 7) (Rose & Van Zyl, 2002). The commercial  $\alpha$ -amylase and endoxylanase were supplied by Anchor Yeast (Johannesburg, South Africa), the commercial endoglucanase was supplied by River Biotech (Cape Town, South Africa) and the pure enzymes were developed by the Department of Microbiology, Stellenbosch University (Stellenbosch, South Africa). The birchwood xylan was purchased from Roth (Karlsruhe, Germany), while the carboxymethyl cellulose (CMC), lichenan, *p*-nitrophenyl  $\beta$ -D-cellobioside (pNPC) and soluble starch were purchased from Sigma-Aldrich (Kempton Park, South Africa). The ethylenediaminetetraacetic acid (EDTA) standard (carbon = 40.99%; nitrogen = 9.57%; hydrogen = 5.56%) and tin foils used during the protein analysis was purchased from Leco Africa (Kempton Park, South Africa). The chemically pure salt and ascorbic acid were purchased from Labchem (Johannesburg, South Africa) and the fresh compressed yeast was obtained from Anchor Yeast (Johannesburg, South Africa). The shortening, used in the baking formulation and to grease the baking tins, was purchased from Chipkins Bakery Supply (Montague Gardens, South Africa) and the soyflour from Impilo Products (Pretoria, South Africa).

### *Determination of moisture, ash and protein contents*

Moisture and ash contents were measured according to the AACC Approved Methods 44-15A and 08-01/2, respectively (AACC, 2000). The protein content was determined with a nitrogen analyser (Truspec<sup>®</sup> N Elemental Determinator, Leco, St. Joseph, Michigan) according to the Dumas combustion method (AACC Approved Method 46-30; AACC, 2000). The analyses were performed in triplicate for every batch of flour.

*Rheological measurements*

A consistograph test was carried out in a Consistograph NG (Tripette et Renaud, France) following the AACC Approved Method 54-50 (AACC, 2000). The parameters recorded were water absorption and tolerance to mixing. The water absorption was also determined using a Brabender Farinograph (Duisburg, Germany). The mixing was carried out at regular speed (63 rpm) at 30°C in a 300 g bowl according to AACC Approved Method 54-21 (AACC, 2000). Additional parameters determined were dough development time (DDT) and dough stability. The viscoelastic behaviour of the dough was determined by the alveograph test, using an Alveograph MA 82 (Chopin, Tripette et Renaud, France) following the AACC Approved Method 54-30A (AACC, 2000). The parameters registered were tenacity or resistance to extension (P), dough extensibility (L), deformation energy (W) and the curve configuration ratio (P/L). The optimum mixing time of the dough was determined using a Mixograph (National Mfg Co., Lincoln, Nebraska, USA) according to the AACC Approved Method 54-40A (AACC, 2000). The presence of  $\alpha$ -amylase activity in the flour was determined using a Shakematic 1095 and a Falling Number 1500 (Perten Instruments AB, Sweden) according to AACC Approved Method 56-81B (AACC, 2000). All the rheological measurements were performed in triplicate for every batch of flour.

*Enzyme activity measurements*

The enzyme activities were measured using dinitrosalicylic acid (DNS) which enabled determination of the amount of reducing sugars released (Miller *et al.*, 1960; Bailey *et al.*, 1992). Endoxylanase activity was measured using 1% birchwood xylan as substrate, while  $\alpha$ -amylase activity was measured using 0.1% unmodified soluble wheat starch. The endoglucanase activity was measured using 1% CMC as the chemical cellulose substrate and 0.1% lichenan as the natural cellulose substrate. The release of reducing sugars was determined after an incubation period of 5 min at 40°C using 0.05 M sodium citrate buffer (pH 5.0). Cellobiohydrolase activity was determined under the same conditions, but using 4 mM *p*NPC as the substrate (Den Haan *et al.*, 2007). The *p*-nitrophenol released from *p*NPC was detected at 405 nm after the addition of 1 mL of 1 M Na<sub>2</sub>CO<sub>3</sub> to raise the pH and terminate the reaction. One unit of enzyme activity was defined as the activity producing 1  $\mu$ mol per min of reducing sugars in xylose/glucose/*p*NP equivalents under these assay conditions. All enzymatic assays were performed in triplicate.

*Bread making process*

The optimised straight-dough bread making method was used as described in AACC Approved Method 10-10B (AACC, 2000). The basic formulation is detailed in **Table 3.1**. The ingredients comprised chemically pure salt, fresh compressed yeast, edible sugar, shortening, soyflour,



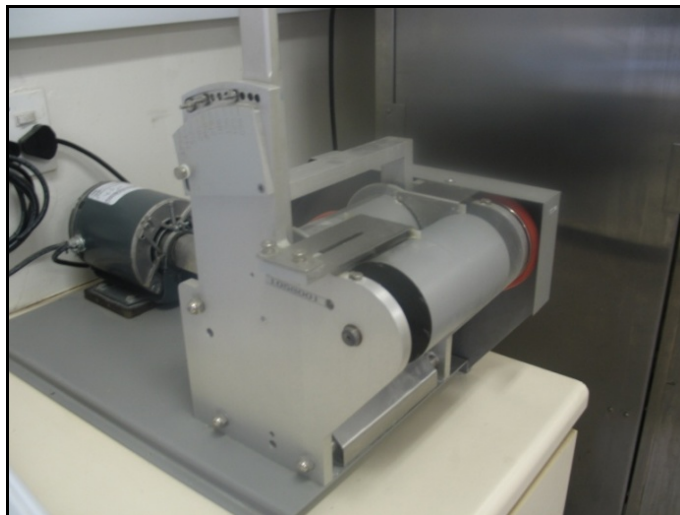
chemically pure ascorbic acid and  $\alpha$ -amylase. The water absorption was optimised for the control sample (based on the moisture and protein contents of the flour) and kept constant for all enzyme treatments. The dough was optimally mixed until dough development, in a pin type mixer (National Manufacturing Company, Lincoln, Nebraska, USA) (**Fig. 3.1**). The average mixing time of the control samples was taken as the standard mixing time which was kept constant for all enzyme treatments. After mixing, the temperature and weight of the dough was recorded and the dough was rounded by hand and placed in a fermentation cabinet to proof for 52 min at 30°C and 85% relative humidity (RH). The dough was passed through a sheeter (National Manufacturing Company, Lincoln, Nebraska, USA) (**Fig. 3.2**) and allowed to ferment for another 25 min. The dough was sheeted and put into well greased pans (141 x 81 x 54 mm) and placed in the fermentation cabinet to proof for 13 min. The dough was sheeted, hand moulded and proofed for 33±2 min. After final proofing (fermentation) the height of the respective loaves were recorded in mm and subsequently baked in a convection oven (Macadams, South Africa) for 20 min at 170°C. The loaves were removed from the pans after baking and weighed immediately. The loaves were left to cool for 1 h at room temperature and the height was measured with a graduated height meter (in cm to the nearest mm) (**Fig. 3.3**). Loaves were placed in plastic bags (Proton Packaging, Paarl) and stored for 24 h at 22°C when quality evaluations (crumb texture, crumb softness and slice brightness) were performed.

**Table 3.1** Details of the basic and adjusted bread making formulations as used during the baking trials

| <b>Ingredients</b>            | <b>Basic formulation</b>       | <b>Adjusted formulation</b>    |
|-------------------------------|--------------------------------|--------------------------------|
|                               | <b>Baking trials 1 &amp; 2</b> | <b>Baking trials 3 &amp; 4</b> |
| Flour (14% m.b.), %           | 100                            | 100                            |
| Distilled water               | Optimum                        | Optimum                        |
| Sugar, %                      | 4                              | 1                              |
| Fresh compressed yeast, %     | 3                              | 1.8                            |
| Shortening (fat), %           | 3                              | 2                              |
| Salt (NaCl), %                | 1.5                            | 2                              |
| Soyflour, %                   | 0.25                           | 0.5                            |
| Ascorbic acid, ppm            | 40                             | 80                             |
| Fungal $\alpha$ -amylase, ppm | 20                             | 7                              |
| m.b. Moisture basis           |                                |                                |



**Figure 3.1** The pin type mixer used to mix dough until dough development.



**Figure 3.2** The sheeter used to sheet the dough.



**Figure 3.3** The graduated height meter used for measuring loaf height (in cm to the nearest mm).

#### *Baking trials for enzyme evaluation*

Four baking trials were conducted. During the first two trials, the bread making formulation according to AACC Approved method 10-10B, (AACC, 2000) was used (referred to as the Basic formulation in **Table 3.1**). Based on results obtained from the first two baking trials this basic formulation was adjusted in order to optimise it for effective enzyme evaluation which was then subsequently used in baking trials 3 and 4 (referred to as the Adjusted formulation in **Table 3.1**). The adjusted formulation consisted of the same ingredients as the basic formulation; with alterations to the concentrations of the ingredients (**Table 3.1**). The activity of the enzymes determined was used to calculate the amount of enzyme to be included in the first baking trial (**Table 3.2**). The enzyme concentrations to be used in the subsequent baking trials (baking trials 2 and 3) were determined based on the results of baking trial 1. The ascorbic acid and all the enzymes were made up to pre-determined concentrations using distilled water. New stock solutions were made daily. The commercial and pure endoxylanases and endoglucanases were also evaluated in combination, respectively. The concentration levels of the enzymes used in this trial (baking trial 4) are given in **Table 3.3**.

**Table 3.2** The concentrations of each enzyme used in baking trials 1, 2 and 3

| Enzymes                  | Concentration (ppm) |                |                      |
|--------------------------|---------------------|----------------|----------------------|
|                          | Basic formulation   |                | Adjusted formulation |
|                          | Baking trial 1      | Baking trial 2 | Baking trial 3       |
| Commercial endoxylanase  | 4                   | 4              | 5                    |
|                          | 15                  | 8              | 12                   |
|                          | 40                  | 15             | 20                   |
| Pure endoxylanase (Xyn2) | 4                   | 4              | 10                   |
|                          | 15                  | 8              | 24                   |
|                          | 40                  | 15             | 40                   |
| Commercial endoglucanase | 24                  | 24             | 3                    |
|                          | 80                  | 40             | 5                    |
|                          | 240                 | 80             | 8                    |
| Pure endoglucanase (EgI) | 40                  | 40             | 40                   |
|                          | 150                 | 80             | 80                   |
|                          | 400                 | 150            | 150                  |

**Table 3.3** The concentrations of each enzyme as used in combination in baking trial 4

| Enzymes       | Concentration (ppm)     |                          |                   |                    |
|---------------|-------------------------|--------------------------|-------------------|--------------------|
|               | Commercial endoxylanase | Commercial endoglucanase | Pure endoxylanase | Pure endoglucanase |
| Combination 1 | 5                       | 5                        | -                 | -                  |
| Combination 2 | 12                      | 3                        | -                 | -                  |
| Combination 3 | 12                      | 5                        | -                 | -                  |
| Combination 4 | -                       | -                        | 10                | 80                 |
| Combination 5 | -                       | -                        | 24                | 40                 |
| Combination 6 | -                       | -                        | 24                | 80                 |

*Evaluation of dough and bread quality*

The overall characteristics of the dough were visually evaluated and compared to those of the control sample after mixing. These included elasticity, surface texture and general handling and moulding of the dough. Any deviations from the control sample were recorded. Physicochemical characteristics of the bread loaves including weight (g), height (cm) and crumb texture were assessed. Crumb texture, slice brightness and crumb softness were not performed during baking trial 1. The texture analysis of the bread was performed after cooling and 24 h of storage at 22°C. The loaves were cut using an adjustable automatic bread slicer (182 Master, Graef, Germany).

Crumb grain characteristics were assessed using a C Cell digital image analysis system (Colibre Control International, Appleton, UK). The analysis was performed on 6 slices obtained from each loaf of bread. The crumb grain characteristics recorded were: total slice area (pixels), maximum height (pixels), number of cells, number of holes and volume of cells and holes; and slice brightness (pixels). Immediately after digital imaging the same slices were used for measuring crumb softness. During baking trial 2 the crumb softness of the loaves were measured in Newton using an Instron Universal Testing Machine (Model 4404, Apollo Scientific, South Africa) on days 1, 3, 5 and 6. Crumb softness, during baking trials 3 and 4, was measured using a Zwick Material Testing Machine (Model Z005, Wirsam, Germany). Both machines were equipped with a 21 mm diameter aluminium cylindrical probe at a cross head speed of 100 mm/s. Two stacked bread slices (25 mm total height) were compressed to a depth of 40%.

### *2.7 Experimental design*

A full-factorial experimental design, performed with four factors at three levels, was used.

### *2.8 Statistical analysis*

Statistical analysis were performed and graphs compiled using STATISTICA version 8 (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was performed to compare average measurements between samples to determine absolute differences. The bar around the average represents the 95% confidence interval for the average measurements. Fisher least significance (LSD) *post-hoc* testing was used. All references to significant differences indicate statistical significant difference at  $P < 0.05$ .

## **Results**

### *Quality attributes of commercial white bread flour*

A new batch of flour was used for each baking trial and the quality attributes of the flour were determined to ensure the flour used was as consistent as possible (**Table 3.4**). The flour used in baking trial 1 had the highest protein content and alveograph distensibility and the shortest mixograph peak time. The flour used in baking trial 2 had the lowest moisture content and therefore the highest mixograph water absorption. The same batch of flour was used in baking trials 3 and 4. This flour had the lowest protein and ash contents, the highest consistograph water absorption, the highest alveograph curve configuration ratio and the longest mixograph peak time.

**Table 3.4** Quality attributes of the flour used in the respective baking trials

| Quality attribute                                | Baking trial 1            | Baking trial 2          | Baking trials 3 and 4   |
|--|---------------------------|-------------------------|-------------------------|
| Moisture, %                                      | 13.9±0.11 <sup>#a\$</sup> | 12.1±0.25 <sup>b</sup>  | 14.0±0.09 <sup>c</sup>  |
| Ash, % (dry weight)                              | 0.93±0.01 <sup>b</sup>    | 0.91±0.01 <sup>a</sup>  | 0.90±0.01 <sup>a</sup>  |
| Protein, % (12% m.b.)                            | 10.6±0.05 <sup>a</sup>    | 10.4±0.05 <sup>a</sup>  | 9.9±0.13 <sup>b</sup>   |
| <u>Consistograph</u>                             |                           |                         |                         |
| Water absorption, % (14% m.b.)                   | 60.0±0.14                 | ND <sup>*</sup>         | 62.2±1.07               |
| Tolerance  | 152±8.48                  | ND                      | 267±25.23               |
| <u>Alveograph</u>                                |                           |                         |                         |
| Tenacity (P), mm                                 | 62±3.05 <sup>b</sup>      | 77±4.93 <sup>a</sup>    | 79±3.21 <sup>a</sup>    |
| Distensibility (L), mm                           | 107±4.04 <sup>b</sup>     | 87±4.04 <sup>a</sup>    | 81±5.03 <sup>a</sup>    |
| Deformation energy (W), x10 <sup>-4</sup> Joules | 171±10.53 <sup>b</sup>    | 201±16.50 <sup>a</sup>  | 203±11.93 <sup>a</sup>  |
| Curve configuration ratio (P/L)                  | 0.58±0.01 <sup>b</sup>    | 0.90±0.08 <sup>a</sup>  | 0.99±0.07 <sup>a</sup>  |
| <u>Farinograph</u>                               |                           |                         |                         |
| Water absorption, % (14% m.b.)                   | 60.5±0.45 <sup>a</sup>    | 59.8±0.3 <sup>b</sup>   | 59.0±0.1 <sup>c</sup>   |
| Dough development time, min                      | 4.2±0.15 <sup>a</sup>     | 3.0±1.73 <sup>a</sup>   | 2.2±0.15 <sup>a</sup>   |
| Stability, min                                   | 4.0±0.32 <sup>a</sup>     | 7.7±0.65 <sup>b</sup>   | 8.6±0.15 <sup>c</sup>   |
| <u>Mixograph</u>                                 |                           |                         |                         |
| Water absorption, %                              | 60.7±0.23 <sup>a</sup>    | 63.76±0.84 <sup>b</sup> | 59.74±0.33 <sup>a</sup> |
| Peak time, min                                   | 2.6±0.04 <sup>a</sup>     | 3.28±0.08 <sup>b</sup>  | 3.52±0.04 <sup>c</sup>  |
| <u>Falling number</u>                            |                           |                         |                         |
| Falling number, s                                | 449±10.69 <sup>a</sup>    | 434±3.31 <sup>b</sup>   | 413±8.50 <sup>c</sup>   |

<sup>#</sup>Mean±SD (standard deviation); <sup>\*</sup>Not determined

<sup>\$</sup>Different letters in the same row indicate significant differences obtained from LSD analysis

### *Enzyme activity measurements*

The activities of the enzymes measured before the trials commenced against different substrates are given in **Table 3.5**. The commercial endoxylanase had activity against the birchwood xylan, the raw starch at 40°C and 60°C and the boiled starch. The pure endoxylanase only had activity against the birchwood xylan. The commercial endoglucanase had activity against all the substrates tested whereas the pure endoglucanase showed activity against CMC, lichenan and birchwood xylan.

**Table 3.5** The activity of the enzymes measured in nkat.g<sup>-1</sup> against selected substrates

| Enzyme                             | CMC <sup>1</sup><br>(40°C) | Lichenan<br>(40°C) | Birchwood xylan<br>(40°C) | Raw<br>starch<br>(40°C) | Raw<br>starch<br>(60°C) | Boiled<br>starch | pNPC <sup>2</sup><br>(40°C) |
|------------------------------------|----------------------------|--------------------|---------------------------|-------------------------|-------------------------|------------------|-----------------------------|
| Commercial<br>endoxylanase         | 0                          | 0                  | 96 222                    | 1427                    | 1767                    | 42 294           | 0                           |
| Pure<br>endoxylanase <sup>3</sup>  | 0                          | 0                  | 103 067                   | 0                       | 0                       | 0                | 0                           |
| Commercial<br>endoglucanase        | 92 556                     | 58 625             | 128 889                   | 1036                    | 1613                    | 9980             | 761                         |
| Pure<br>endoglucanase <sup>4</sup> | 16 178                     | 26 854             | 2978                      | 0                       | 0                       | 0                | 0                           |

<sup>1</sup>Carboxymethyl cellulose; <sup>2</sup>p-nitrophenyl β-D-cellobioside; <sup>3</sup>*T. reesei* Xyn2; <sup>4</sup>*T. reesei* EgI

### *Evaluation of dough and bread quality*

#### Dough quality

Compared to the control, the commercial endoxylanase produced a slightly sticky, softer dough while the pure endoxylanase produced a slightly dry, firmer dough. The commercial endoglucanase influenced dough characteristics considerably and resulted in a very sticky dough that was difficult to handle, sheet and mould. The concentration at which this enzyme was used had to be decreased in subsequent baking trials, until optimum dough characteristics were obtained. The pure endoglucanase did not influence the dough characteristics. The respective commercial and pure enzymes were combined in baking trial 4 and the commercial endoxylanase and endoglucanase combination resulted in a sticky dough. This was observed when the medium concentrations (12 and 3 ppm, respectively) of the respective enzymes were used. The pure endoxylanase and endoglucanase could be combined in any of the three concentration levels without having a detrimental influence on the dough characteristics.

#### Bread weight

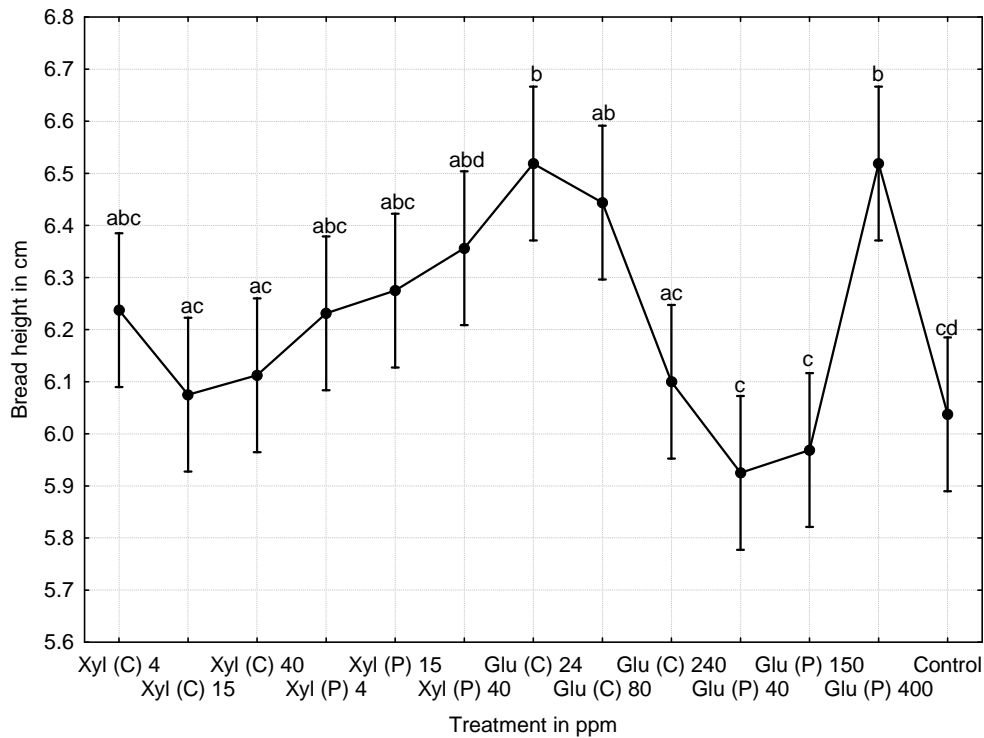
The weight of each loaf was measured immediately after baking. No significant ( $P>0.05$ ) differences in bread weight between the treatments and the control were observed for baking trials 1-3 (results not shown). There were also no significant ( $P>0.05$ ) differences in bread weight between the treatments and the control when combinations of the respective pure and commercial endoxylanases and endoglucanases were evaluated (baking trial 4) (results not shown).

#### Bread height

According to the results of trial 1, the commercial endoglucanase improved bread height significantly ( $P<0.05$ ) at 24 and 80 ppm, while the pure endoglucanase improved bread height

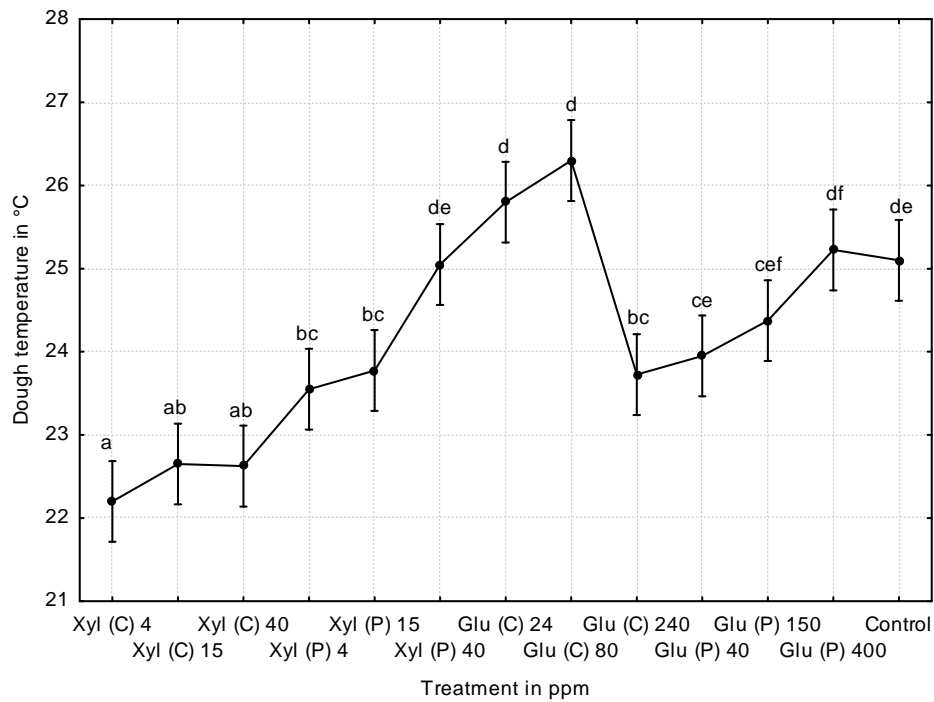
significantly ( $P<0.05$ ) at 400 ppm compared to the control (**Fig. 3.4**). The temperature of the dough was measured after mixing and fluctuations in the temperature were observed (**Fig. 3.5**). In general the endoglucanases had higher dough temperatures compared to the other treatments.

According to the results of trial 2, it seems as if the enzymes did not cause a significant ( $P>0.05$ ) increase in bread height when measured manually with the height meter (**Fig. 3.6**). However, when the bread height was measured by means of digital imaging using the C Cell (**Fig. 3.7**) the results showed tendencies towards increased bread height for the commercial and pure endoxylanases compared to the control, although not significant ( $P>0.05$ ). The results shown are the averages for all the concentrations of each enzyme. **Figures 3.8** shows examples of loafs where individual enzymes are compared to the control. **Figure 3.9** shows digital images obtained from the C Cell to measure bread height.

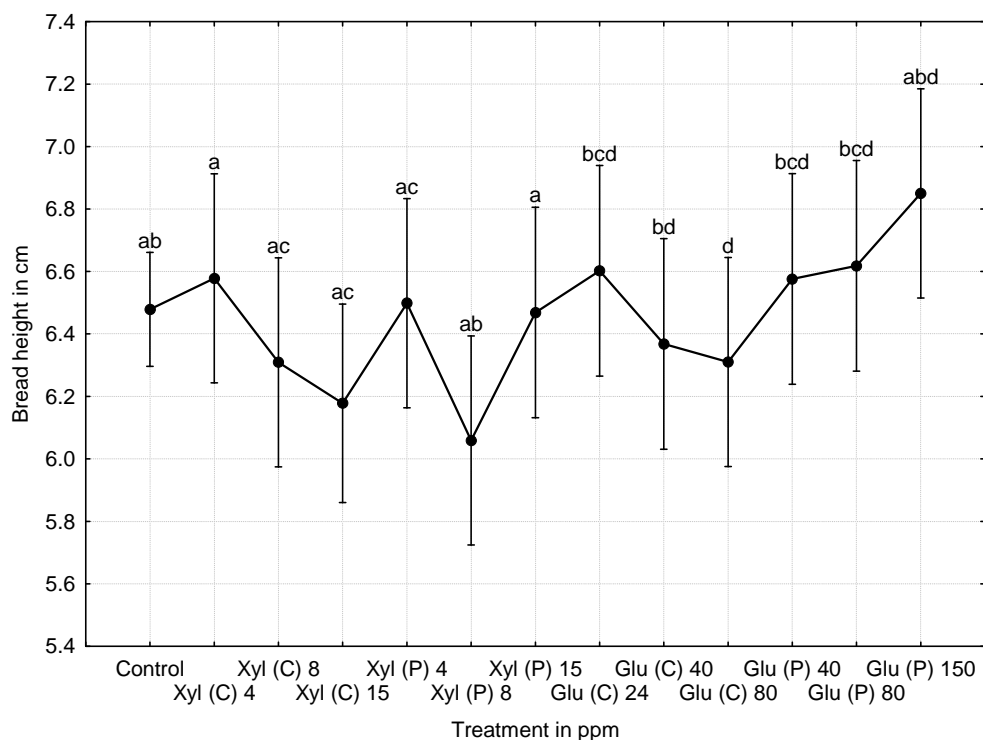


**Figure 3.4** Differences between the average bread heights of trial 1 as determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

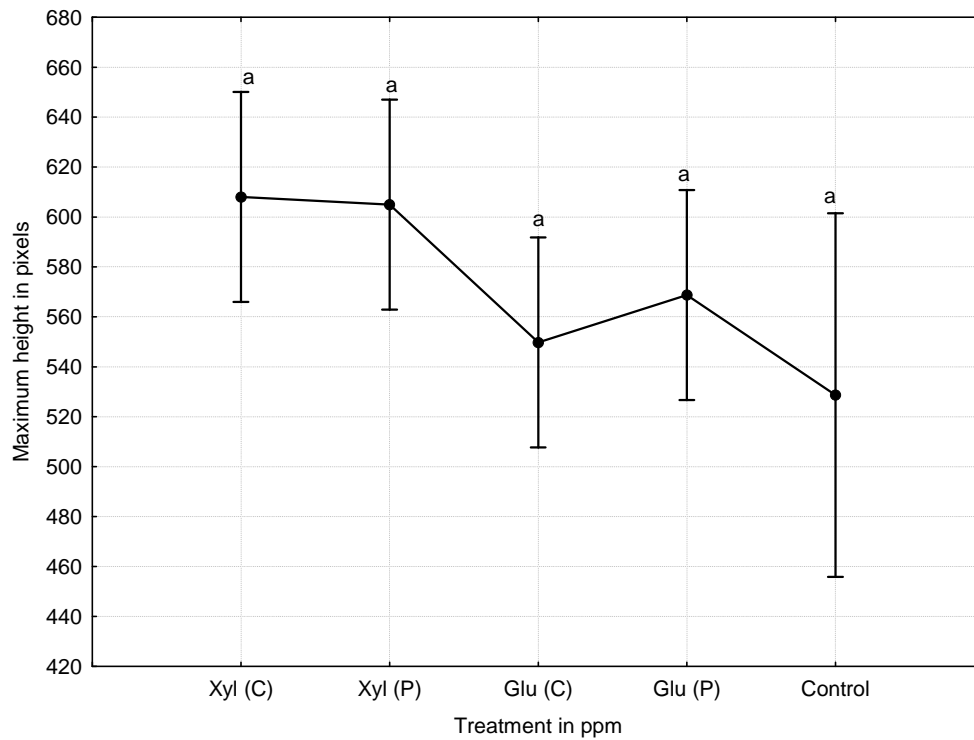




**Figure 3.5** Differences between the average dough temperature of trial 1 as determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



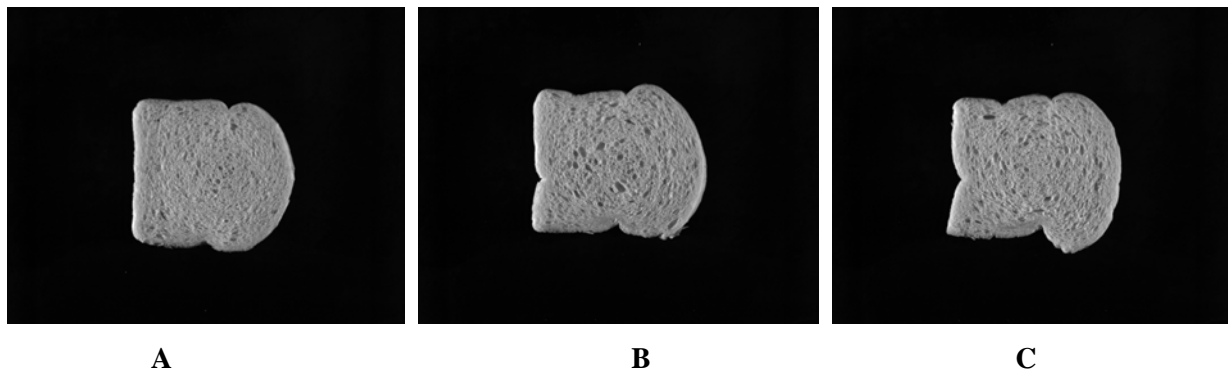
**Figure 3.6** Differences between the average bread heights (manually) of trial 2 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.07$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 3.7** Differences between the average bread heights (digital imaging), of trial 2. The results shown are the average for all the concentrations of each enzyme and were determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.13$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 3.8** Control compared to the commercial endoxylanase at 4 ppm and the pure endoglucanase at 4 ppm.



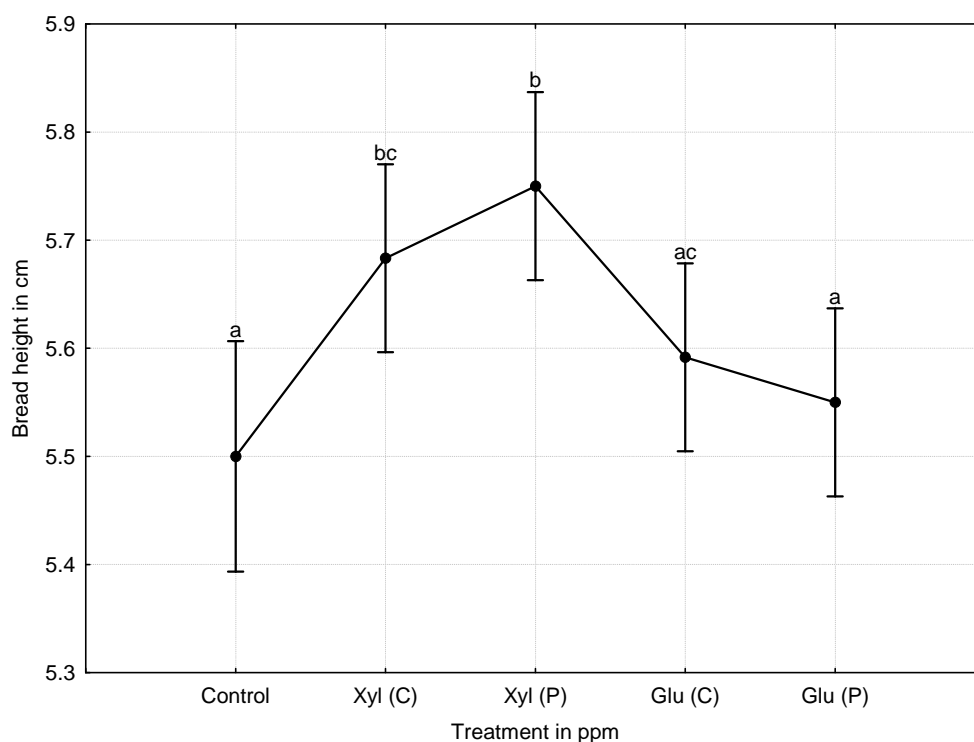
A

B

C

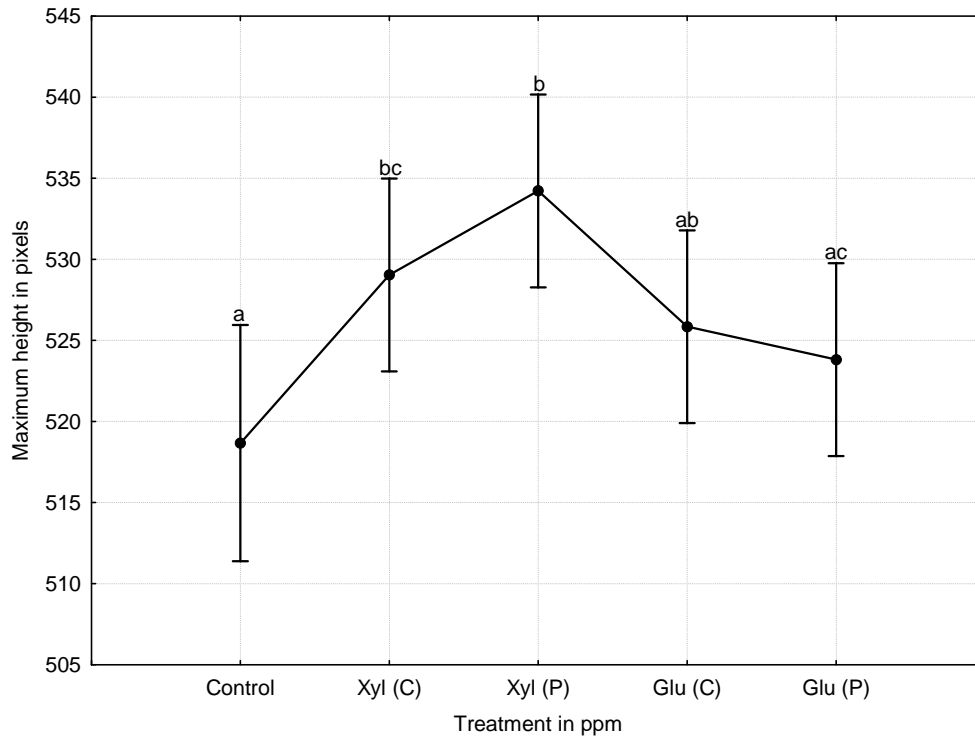
**Figure 3.9** Digital images obtained from the C Cell were the control (A) is compared to the commercial endoxylanase at 20 ppm (B) and the pure endoxylanase at 40 ppm(C).

In baking trial 3, the bread height measured manually (**Fig. 3.10**) and by digital imaging using the C Cell (**Fig. 3.11**) gave similar results. Both the commercial and pure endoxylanases significantly ( $P<0.05$ ) increased bread height compared to the control. The results shown in **Fig. 3.10** and **3.11** are the averages for all the concentrations of each enzyme. Comparing the different enzyme concentrations (**Fig. 3.12**), it was observed that similar results were obtained with the highest concentration of the commercial endoxylanase (20 ppm) and the lowest (10 ppm) and highest (40 ppm) concentration of the pure endoxylanase, all resulting in significantly higher ( $P<0.05$ ) bread heights compared to the control.

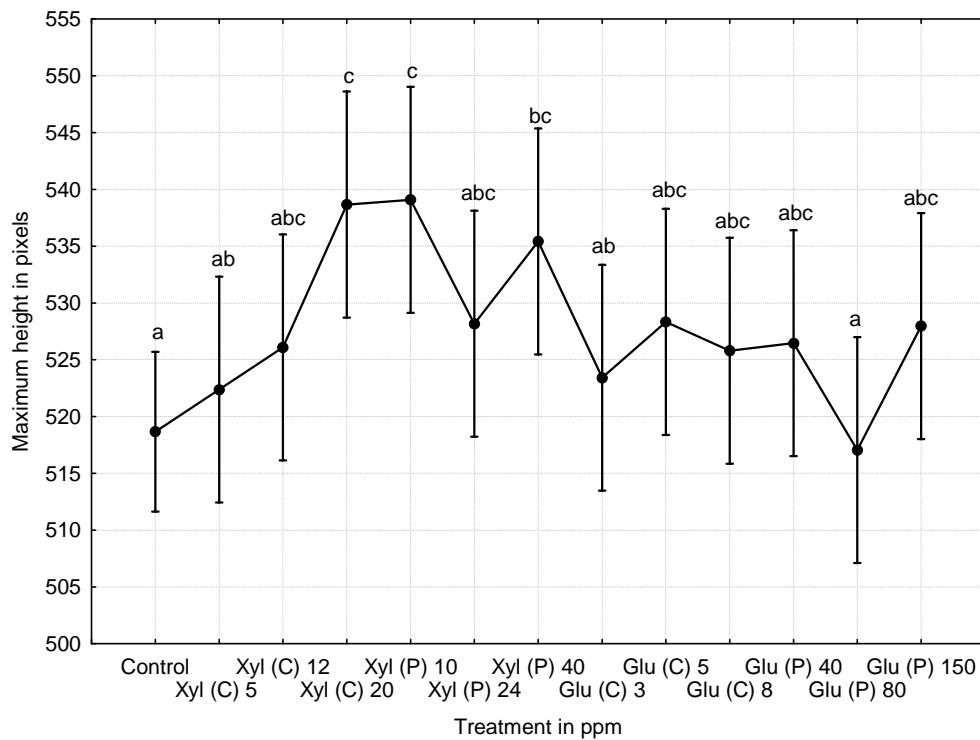


**Figure 3.10** Differences between the average bread heights (manually) of trial 3 (concentrations averaged) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

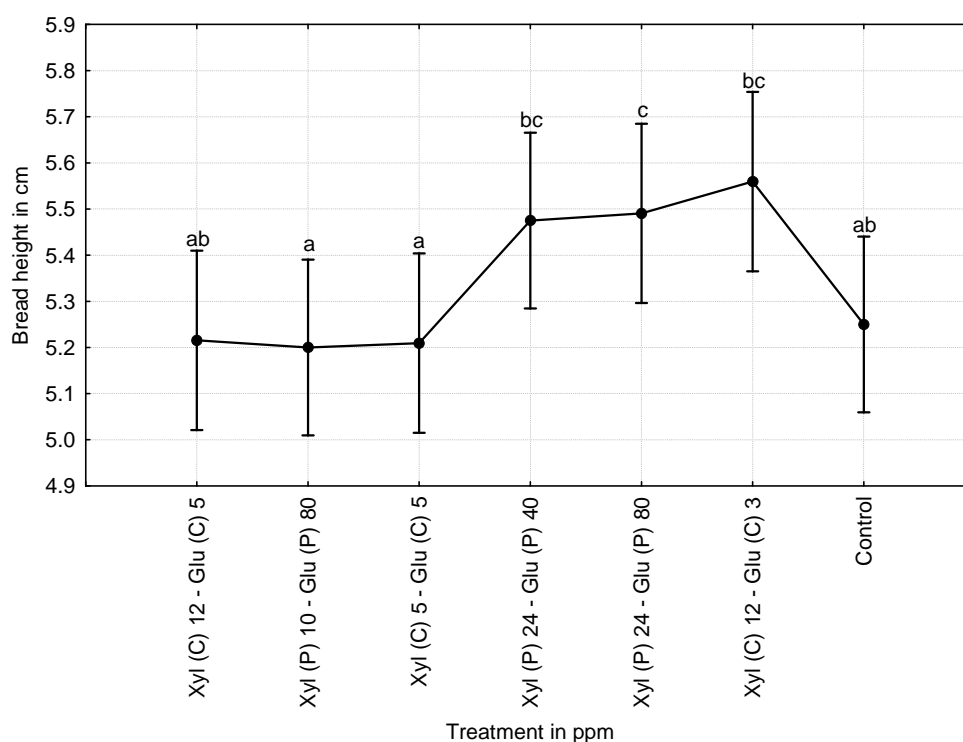
The enzymes were evaluated in combinations during baking trial 4. According to the manual bread height measurements (**Fig. 3.13**) only one combination significantly ( $P<0.05$ ) increased bread height. This was the pure endoxylanase at 24 ppm combined with the pure endoglucanase at 80 ppm. However, according to the C Cell bread height measurements (**Fig. 3.14**) three of the enzyme combinations significantly ( $P<0.05$ ) increased bread height. These combinations included commercial endoxylanase (12 ppm) and endoglucanase (3 ppm); pure endoxylanase (24 ppm) and endoglucanase (40 ppm); as well as pure endoxylanase (24 ppm) and endoglucanase (80 ppm). The pure enzymes could be used at higher concentrations without influencing the dough or bread characteristics detrimentally.



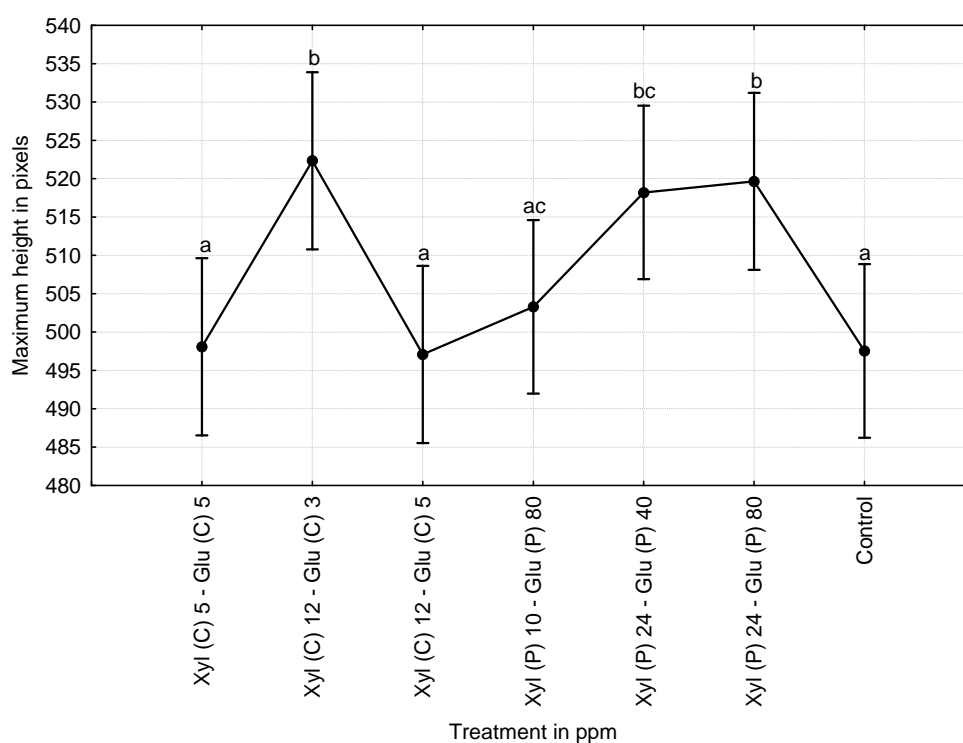
**Figure 3.11** Differences between the average bread heights (digital imaging) of trial 3 (concentrations averaged) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 3.12** Differences between the average bread heights (digital imaging) of trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



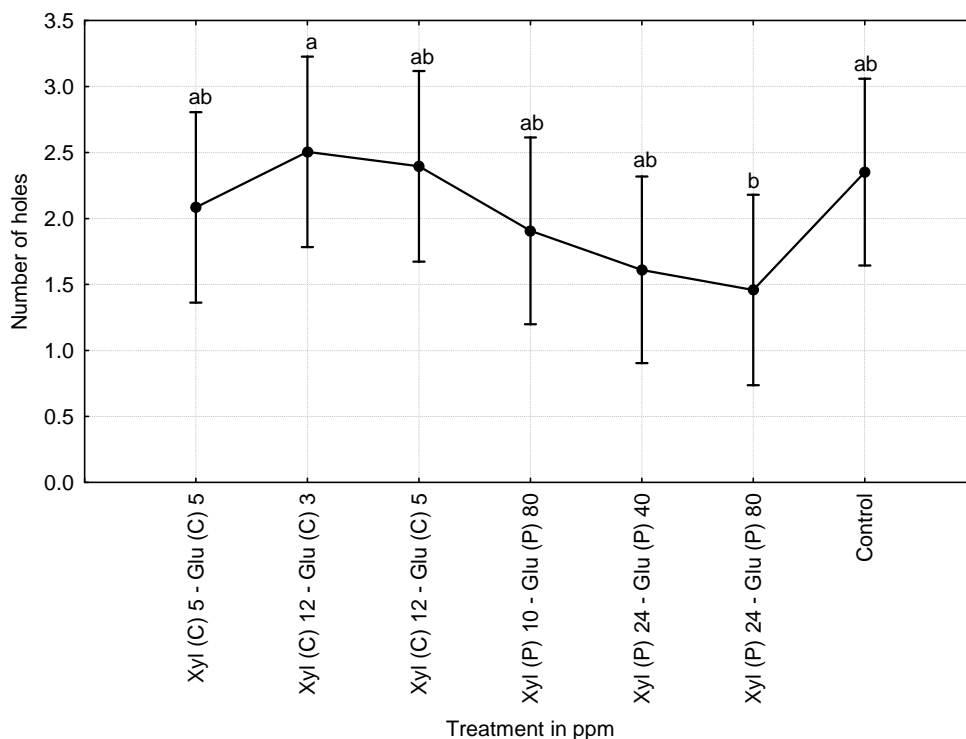
**Figure 3.13** Differences between the average bread heights (manually) of trial 4 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



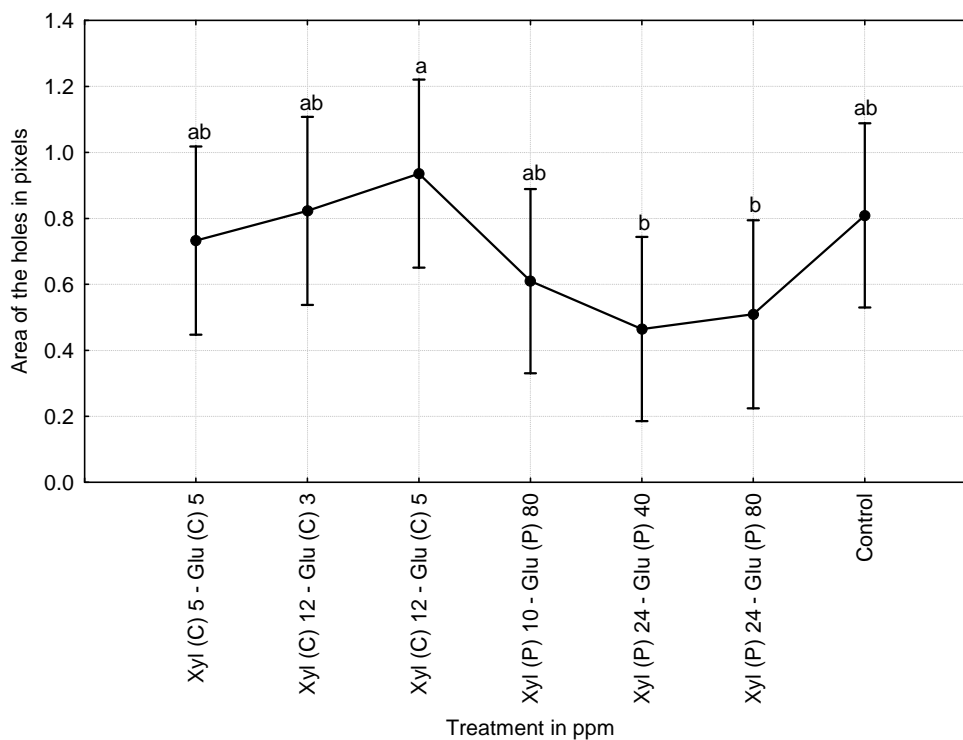
**Figure 3.14** Differences between the average bread heights (digital imaging) of trial 4 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

### Bread crumb texture

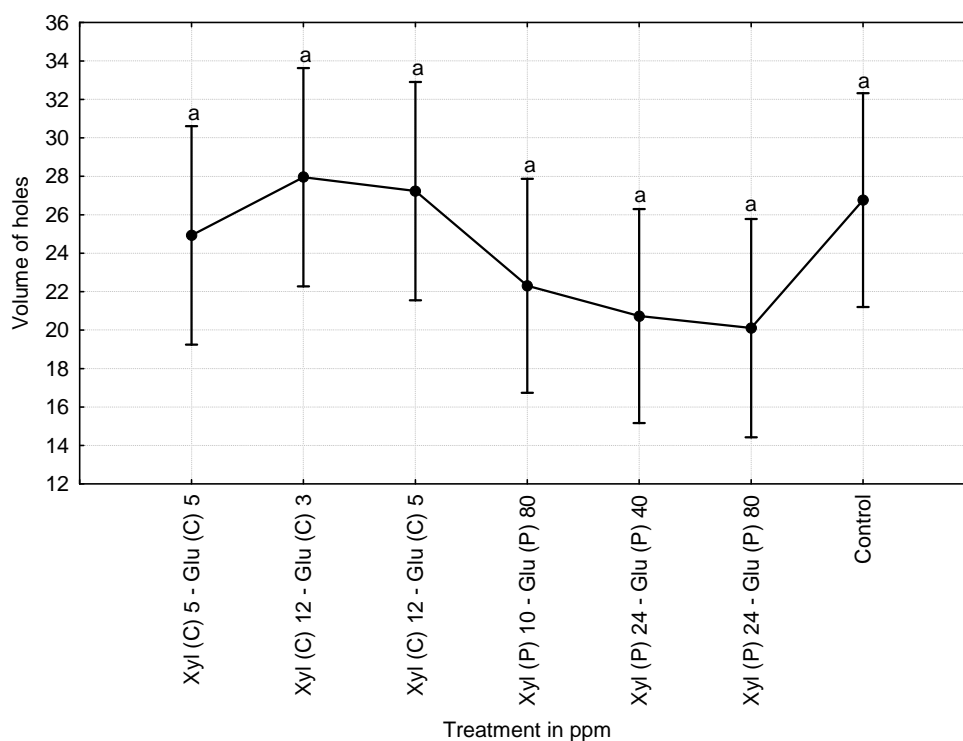
When evaluating the bread crumb texture with the C Cell, the most important measurements considered were the number of cells, number of holes and the area and volume of each. An increase in the number of cells and a decrease in the number of holes, size and volume of the cells and holes would lead to a finer texture. The crumb texture was not measured during trial 1. None of the treatments used during trials 2 and 3 had a significant ( $P>0.05$ ) influence on bread crumb texture (results not shown). From the results of baking trial 4, tendencies could be seen, although these results were not significant ( $P>0.05$ ) (**Figs. 3.15-3.17**). All the combinations of the pure enzymes seemed to decrease the number of holes, area of the holes, as well as the volume of the holes.



**Figure 3.15** Differences between the average number of holes of trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.26$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 3.16** Differences between the average area of holes in trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.17$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

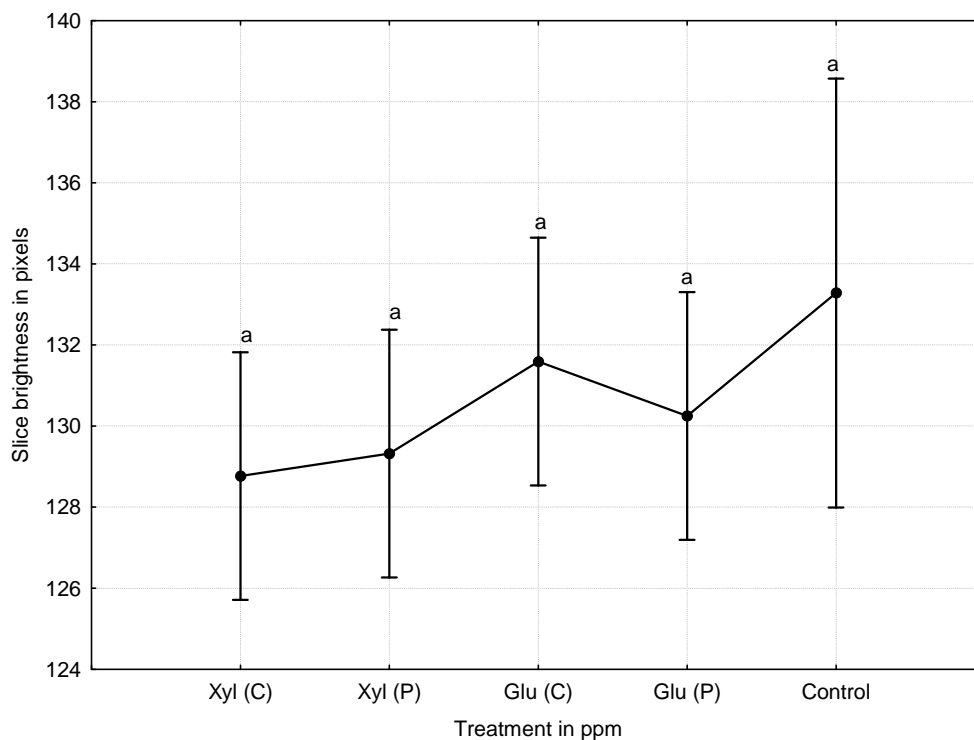


**Figure 3.17** Differences between the average volume of holes in trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.24$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



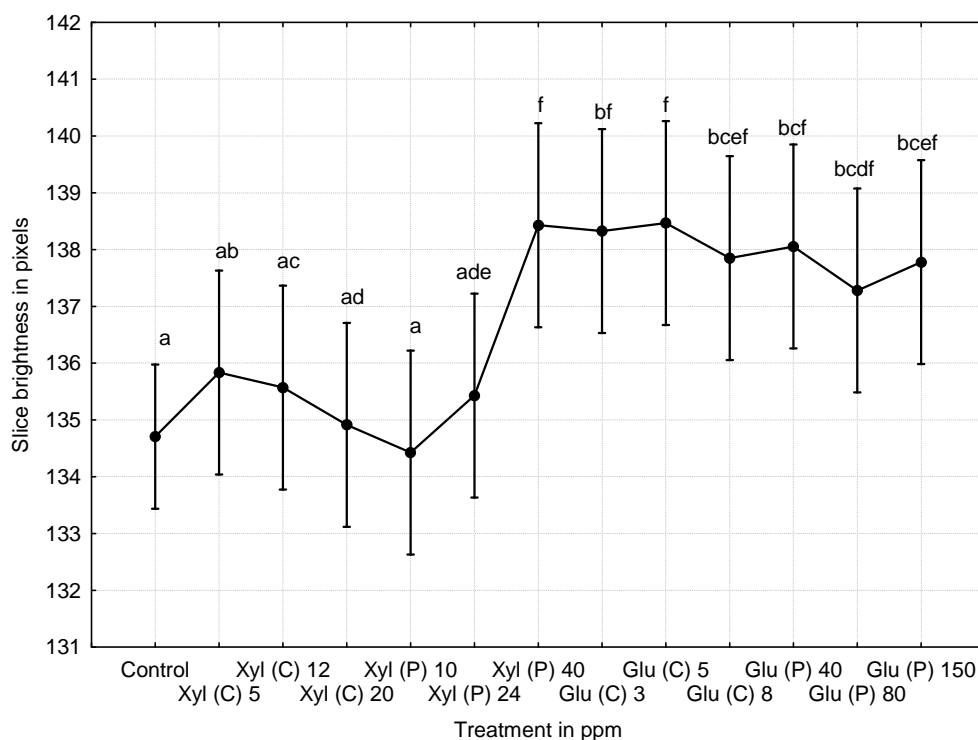
### Slice brightness

The brightness of each slice was measured using the C Cell and results are given in pixels. The slice brightness of trial 1 was not measured. According to the results of baking trial 2 there was no significant ( $P>0.05$ ) increase in slice brightness compared to the control (**Fig. 3.18**). The results shown are averages of all the concentrations of each enzyme. It seemed as if the slices of the control loaves were slightly brighter than those containing the added enzymes.



**Figure 3.18** Differences between the average slice brightness of trial 2. The results shown are the average of the concentrations of each enzyme determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis ( $P=0.41$ ) (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

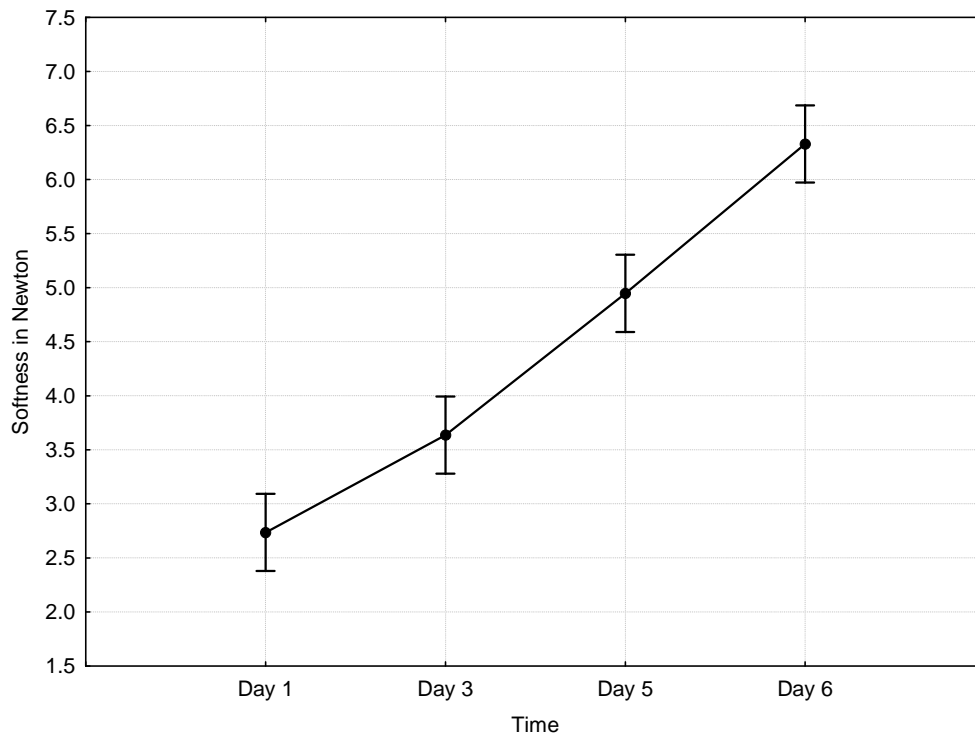
During baking trial 3, the commercial and pure endoglucanases significantly ( $P<0.05$ ) increased slice brightness compared to the control (**Fig. 3.19**). The commercial endoglucanase significantly ( $P<0.05$ ) increased slice brightness at all three concentrations used (3, 5 and 8 ppm). Similar results were observed for the pure endoglucanase which also significantly ( $P<0.05$ ) increased slice brightness at all three enzyme concentrations (40, 80 and 150 ppm). When the pure endoxylanase was used at the highest concentration (40 ppm), the slice brightness was significantly ( $P<0.05$ ) increased. When the enzymes were combined and compared in baking trial 4 there was no significant ( $P>0.05$ ) increase in slice brightness compared to the control (results not shown).



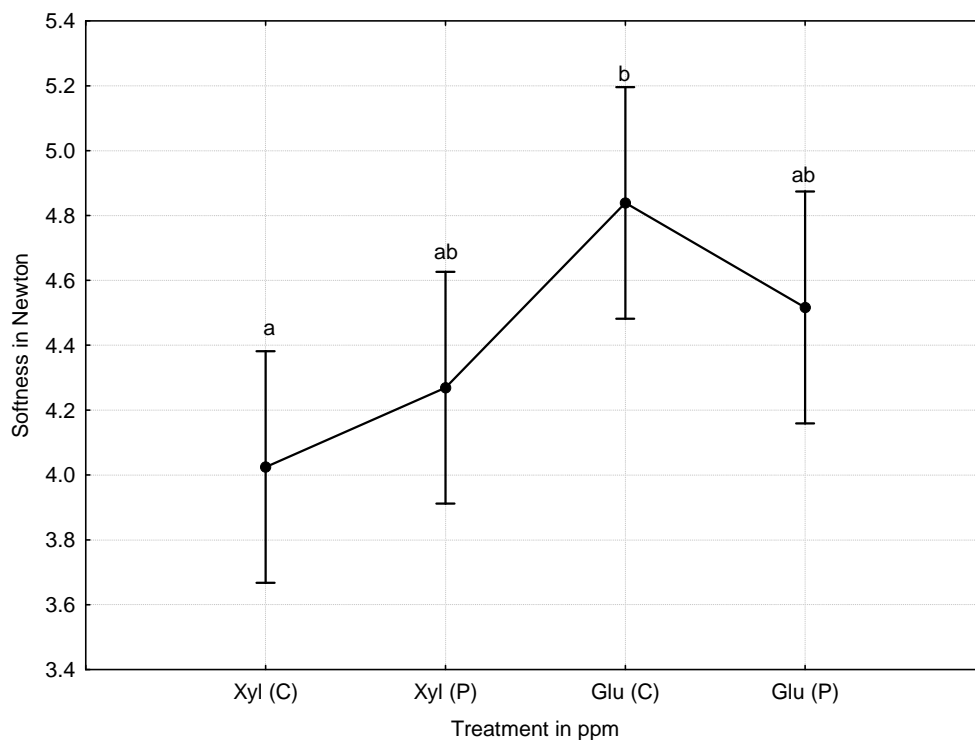
**Figure 3.19** Differences between the average slice brightness of trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

### Crumb softness

In baking trial 1, the crumb softness was measured over time and as expected there was a gradual increase in firmness over time (**Fig. 3.20**). From baking trial 2, it seemed as if both the commercial and pure endoxylanases led to an increase in softness compared to the respective endoglucanases (**Fig. 3.21**). The results shown are the averages of all the concentrations of each enzyme. Only one repetition of each treatment was tested on each of the four days and therefore these results could not be analysed statistically. In addition it was not possible to include the control in the statistical analysis.



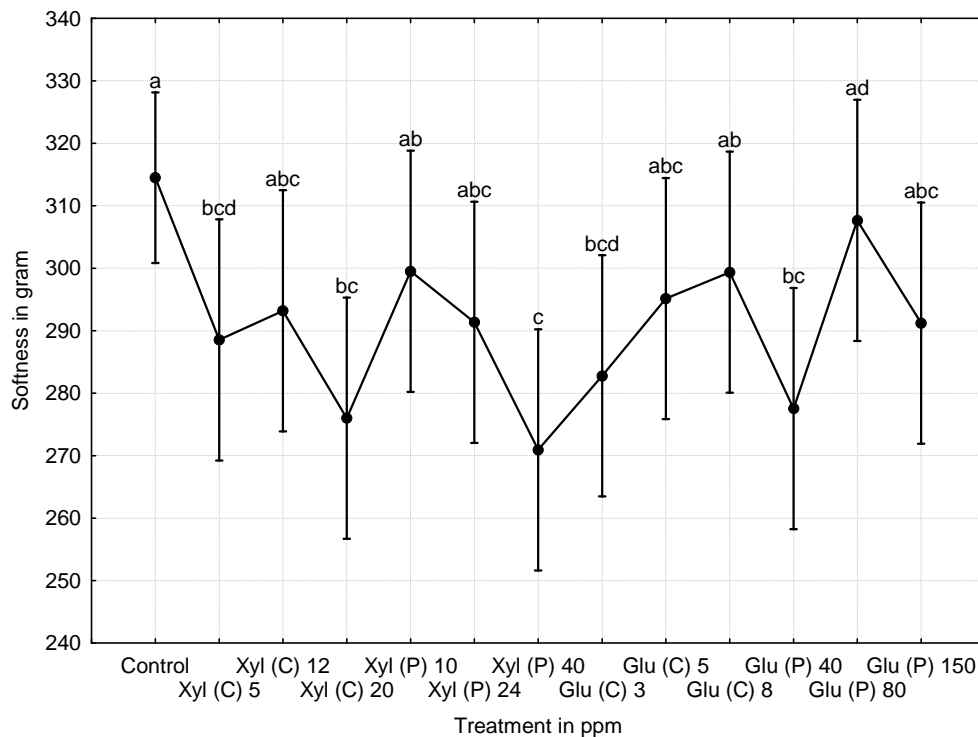
**Figure 3.20** Differences between the average crumb softness of trial 2 measured over time determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals.



**Figure 3.21** Differences between the average crumb softness of trial 2. The results shown are the averages of the concentrations for each enzyme and were determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

When the commercial endoxylanase was used at 5 and 20 ppm it significantly ( $P<0.05$ ) increased crumb softness (**Fig. 3.22**). The pure endoxylanase significantly ( $P<0.05$ ) increased crumb softness, when it was used at 40 ppm. The lowest concentration of both the commercial (3 ppm) and the pure endoglucanases (40 ppm) led to an increase in crumb softness measured on day 1. The highest concentration (40 ppm) of the pure endoxylanase gave the softest crumb on day 1.

The enzymes were evaluated in combinations in baking trial 4 and the softness of the bread crumb was evaluated on day 1. No significant ( $P>0.05$ ) differences were observed compared to the control (results not shown).



**Figure 3.22** Differences between the average crumb softness of trial 3 determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

## Discussion

### *Quality attributes of commercial white bread flour*

The differences observed in the quality of the respective batches of flour were compensated for by optimising the water content for the control sample (based on the moisture and protein contents of the flour). This was then kept constant for all enzyme treatments. The dough was also optimally mixed and the average mixing time of the control samples was taken as the standard mixing time which again was kept constant for all enzyme treatments.

### *Enzyme activity measurements*

The commercial endoxylanase was able to degrade birchwood xylan as well as the starch; hence containing both endoxylanase and  $\alpha$ -amylase activities. The pure endoxylanase (Xyn2) only displayed activity towards birchwood xylan. The pure endoxylanase had a slightly higher xylanase activity than the commercial endoxylanase as a result of different concentrating methods. The commercial endoglucanase was able to break down all the substrates indicating the presence of various enzymes. This enzyme displayed high levels of endoglucanase activity as it was able to break down CMC (a chemical cellulose substrate) as well as lichenan (a natural cellulose substrate). It also contained endoxylanase activity,  $\alpha$ -amylase activity and cellobiohydrolase activity as it was able to break down birchwood xylan, starch and *p*-nitrophenyl  $\beta$ -D cellobioside, respectively. The commercial endoglucanase also displayed more xylanase activity than any of the other endoxylanases tested. The pure endoglucanase (EgI) was able to break down CMC, lichenan and birchwood xylan and therefore displayed endoglucanase as well as some endoxylanase activity. This is not indicative of a mixture of enzymes as the EgI of *T. reesei* is known for its ability to degrade a wide range of different substrates (Bailey *et al.* 1992).

### Dough quality

The commercial endoxylanase produced a slightly sticky and softer dough compared to the control. In a study conducted by Hille (2005), the same enzyme was used in baking trials and it produced a dough that was extensible and sticky after mixing, but recovered well. It was also reported that the use of endoxylanases had a softening effect on dough (Caballero *et al.*, 2006; Selinheimo *et al.*, 2006). In another study dough containing high levels of fungal endoxylanases was sticky and slack immediately after mixing, whereas dough containing bacterial endoxylanases was dry and more consistent (Hile & Schooneveld-Bergmans, 2004). In this study the pure endoxylanase derived from *Trichoderma* sp. produced a slightly dry and firmer dough. This was also observed by Hille (2005) when the doughs containing a bacterial endoxylanase felt dry after mixing and during further processing. In contrast the commercial endoglucanase resulted in a very sticky dough that was

difficult to handle, sheet and mould. This enzyme was able to break down all the substrates tested for in the enzyme activity assay. This may have resulted in too much water released from the arabinoxylans as this enzyme had more endoxylanase activity ( $\text{nkcat.g}^{-1}$ ) than the commercial or pure endoxylanases. Although the AX content was not determined in this study it is known that the AX content of white bread flour is 2-3% (Baillet *et al.*, 2003). It is further possible that excessive starch degradation took place as this enzyme showed  $\alpha$ -amylase activity when tested against the boiled starch. The concentration levels of this enzyme thus had to be decreased. Because of this enzymes' ability to break down a wide range of substrates it should be used at lower concentration levels. All these factors could contribute to sticky dough that was obtained after mixing and during further processing.

### *Baking trial 1*

From the results of the enzyme activity measurements ( $\text{nkcat.g}^{-1}$ ) (**Tables 3.5 & 3.6**) the concentrations for each enzyme was calculated. These calculations were based on the recommended concentration levels to be used as obtained from the supplier. The endoxylanase activity of the commercial and pure endoxylanases was shown to be similar in the first enzyme activity assay. Therefore, they were used at the same concentration levels in baking trial 1.

As expected there were no significant ( $P>0.05$ ) differences in bread weight. Significant differences in bread weight would have indicated mistakes made when the ingredients or the dough were weighed. As no significant differences were observed in bread weight, the bread making process was therefore confirmed to be accurate and repeatable.

From the results of baking trial 1, it is observed that the endoglucanases significantly ( $P<0.05$ ) increased bread height at different concentrations (**Fig. 3.4**). From other studies it is known that endoglucanases can result in an increase in bread height (Harada *et al.*, 2000; 2005), but they are known to increase bread height to a lesser extent compared to endoxylanases (Dr. J.D.R. Hille, Product Development Manager, DSM Baking Enzymes, Delft, Netherlands, personal communication, 2008). When the dough temperatures were measured, fluctuations in temperature were observed and the doughs containing endoglucanases in general had higher dough temperatures than the doughs containing endoxylanases (**Fig. 3.5**). These higher temperatures would have enabled the dough to have higher proof heights as well as subsequent bread heights. Due to the temperature fluctuations of the doughs these results therefore needed to be confirmed in terms of the effects of the enzymes on bread quality characteristics.

*Baking trial 2*

The actual concentrations used in baking trial 1 (4, 15 and 40 ppm), determined based on the measured enzyme activities, were higher than recommended by the suppliers. As no significant results were obtained and sticky doughs were observed during baking trial 1, the concentration levels for both the commercial and pure endoxylanases were decreased in baking trial 2 (4, 8 and 15 ppm). Due to very sticky doughs observed during baking trial 1, the levels of the commercial and pure endoglucanase were decreased for baking trial 2. According to Hille (2005) the optimum concentration for a cellulase enzyme is 100 ppm. This recommended concentration was much lower than the initial concentrations used (based on measured enzyme activity) for the commercial (24, 80 and 240 ppm) and the pure endoglucanases (40, 150 and 400 ppm).

After conducting several preliminary baking trials as well as baking trials 1 and 2, still only tendencies in terms of bread quality characteristics, were seen, but no significant ( $P>0.05$ ) results could be obtained. According to literature cited (Hammer, 1995; Poutanen, 1997; Popper, 1997; Martinez-Anaya & Jimenez, 1997; Harada *et al.*, 2000; Courtin *et al.*, 1999, 2001; Hille & Schooneveld-Bergmans, 2004; Jiang *et al.*, 2004; Hille, 2005; Harada *et al.*, 2005; Caballero *et al.*, 2006; Shah *et al.*, 2006; Dr. J.D.R Hille, Product Development Manager, DSM Baking Enzymes, Delft, the Netherlands, personal communication, 2008) the use of both endoxylanases and endoglucanases should result in improved dough and bread quality characteristics. Anchor Yeast (Lorraine Bezuidenhout, Business Manager, Cereal Enzymes, Anchor Yeast, Johannesburg, South Africa) was thus approached who tested the effect of the commercial enzymes using a commercial bread making formulation. It was observed that the commercial enzymes improved loaf height, when this commercial formulation was used. The bread baking formulation used during baking trials 1 and 2 was evaluated and compared to a commercial white bread formulation. The formulation to be used in subsequent baking trials was adjusted accordingly (Dr. J.D.R Hille, Product Development Manager, DSM Baking Enzymes, Delft, Netherlands, personal communication, 2008). The adjustments were only in terms of concentration levels of the ingredients used.

The basic formulation contained high levels of sugar, yeast, fat and  $\alpha$ -amylase (**Table 3.1**). Sugar is used as a nutrient by the yeast which produces carbon dioxide and results in an increase in loaf volume (Chung & Park, 1997). Fat crystals stabilises gas cells which allows them to expand during baking without rupturing and produces bread with high loaf volume (Brooker, 1996). The  $\alpha$ -amylases facilitate maltose production in combination with the endogenous  $\beta$ -amylases by degrading the damaged starch particles during the dough stage and generating low molecular weight dextrans. The maltose released can then be used by the yeast as a fermentable sugar (Linko *et al.*, 1997). Treatment with  $\alpha$ -amylase lowers the viscosity of the dough, improves handling and results



in a softer and larger loaf of bread (Bowles, 1996) Therefore the levels of these ingredients were decreased in order to enable the effect of the different enzymes on bread quality characteristics to be more prominent.

The levels of salt, soyflour and ascorbic acid were found to be too low. Salt (Hoseney, 1986) and ascorbic acid (Kent & Evers, 1994) are known to strengthen the gluten and therefore the levels of salt and ascorbic acid were increased. The level of soyflour was also increased as it is known that the addition of enzyme active soyflour leads to an improved crumb texture and crumb colour (Figoni, 2004). The adjusted formulation could subsequently be considered as a lean formulation which can be used to study the effect of ingredients or enzymes on bread baking quality. This adjusted formulation was used when conducting baking trials 3 and 4 which resulted in the effects of the enzymes to be more prominently observed.

As expected no significant ( $P>0.05$ ) differences in bread weight were obtained. From the measurements performed with the manual height meter (**Fig. 3.6**) as well as the C Cell (**Fig. 3.7**), it is observed that the enzymes did not have a significant ( $P>0.05$ ) effect on the bread height in baking trial 2. However, from the C Cell measurements tendencies could be seen. It seemed as if the endoxylanases could lead to a higher increase in loaf height than the endoglucanases, but these results were not significant ( $P>0.05$ ). There were not enough repetitions of each treatment and this may have contributed to the results not being significant.

From the results of baking trial 2, it is observed that the enzymes had no significant ( $P>0.05$ ) influence on slice brightness (**Fig. 3.18**). This can be attributed to the insufficient number of repetitions, the baking formulation used and the fact that the optimum concentration levels were not yet established. According to the results of baking trial 2 there was a gradual decrease in softness over time as the bread stales and loses its freshness (**Fig. 3.20**). It also seemed as if the endoxylanases increased the softness of the bread crumb on day 1 (**Fig 3.21**). There was only one repetition of each treatment evaluated for softness and therefore the control could not be included in the statistical analysis and could not be used for comparison.

### *Baking trial 3*

The concentrations of the commercial and pure endoxylanases were slightly increased in baking trial 3, because the literature cited showed that the optimum concentration for fungal endoxylanase was indicated to be 20 ppm and 40 ppm for bacterial endoxylanase (Hille, 2005). This was carried out in order to obtain the optimum concentration level of each enzyme. The use of the commercial endoglucanase resulted in a slack and sticky dough that was difficult to handle. Therefore the level of the commercial endoglucanase was decreased from baking trial 2 (24, 40 & 80 ppm) to baking trial 3 (3, 5 & 8 ppm) in order to find the optimum concentration.

In baking trial 3 the adjusted formulation was used (**Table 3.1**). No significant ( $P>0.05$ ) differences in bread weight were obtained. For bread height, the manual measurements (**Fig. 3.10**) and the C Cell measurements (**Fig. 3.11**) gave similar results. This indicates that the manual measurements are indeed very accurate. When considering the average values for bread height it is observed the commercial and the pure endoxylanases significantly ( $P<0.05$ ) increased bread height compared to the control and the loaves containing endoglucanases (**Figs. 3.10 & 3.11**). Endoxylanases are known to increase loaf height (Hammer, 1995; Poutanen, 1997; Courtin *et al.*, 1999, 2001; Hille & Schooneveld-Bergmans, 2004; Hille, 2005; Shah *et al.*, 2006). The commercial endoxylanase derived from *A. niger* and the pure endoxylanase derived from *Trichoderma* sp. belongs to the GH family 11 which is known to have preference for water un-extractable arabinoxylans (WU-AX) (Hille, 2005). The WU-AX can hold up to ten times their weight in water (Kim & D'Appolonia, 1977). Therefore, the increase in bread height/volume can be attributed to the ability of the endoxylanases to hydrolyse the water un-extractable part of the arabinoxylans (AX). As a result of the hydrolysis the previously bound water is released and redistributed to other components of the dough. Damaged starch and gluten will take up the water and used it for starch gelatinisation and gluten formation, respectively. This results in improved extensibility of the gluten, gas retention and reduced resistance to rise (Hille, 2005). The WU-AX also destabilises dough structure and are present in fragments that can form physical barriers to the gluten network during dough development (Courtin & Delcour, 2002). The hydrolysis will lead to a more stable dough as the gluten will be able to hold more carbon dioxide released during yeast fermentation and this will result in improved proof height, bread height and/or volume.

It seemed as if the pure endoxylanase may have caused a larger increase in bread height compared to the commercial endoxylanase. When looking at the effect of each specific concentration on bread height it is interesting to note that the commercial endoxylanase had to be used at a higher concentration than the pure endoxylanase to obtain the same increase in bread height.

The results of baking trial 3 showed that the pure and the commercial endoglucanases significantly ( $P<0.05$ ) increased slice brightness measured on day 1 (**Fig. 3.19**). This increase in brightness also seemed to be irrespective of the concentrations used. Endoglucanases are known to improve dough characteristics, crumb texture and crumb softness (Dr. J.D.R Hille, Product Development Manager, DSM Baking Enzymes, Delft, the Netherlands, personal communication, 2008). When the pure endoxylanase was added at the highest concentration, slice brightness was significantly ( $P<0.05$ ) increased. It could be possible that the addition of these enzymes may cause smaller or shallower cells and/or holes and this will lead to a finer crumb texture with smaller shadows on the bread slice which can cause an increase in overall slice brightness. The number,

area or volume of the cells or holes were not significantly ( $P>0.05$ ) decreased. According to baking trials 2 and 3 none of the treatments used had a significant ( $P>0.05$ ) influence on the texture of the bread crumb.

Both the commercial and pure endoxylanases significantly ( $P<0.05$ ) increased softness at the different concentrations used (**Fig. 3.22**). These results are similar to studies where endoxylanases used at optimum levels was also shown to improved crumb softness (Hammer, 1995; Martinez-Anaya & Jimenez, 1997; Popper, 1997; Poutanen, 1997; Harada *et al.*, 2000; Hille & Schooneveld-Bergmans, 2004; Jiang *et al.*, 2004; Harada *et al.*, 2005; Hille, 2005; Caballero *et al.*, 2006; Shah *et al.*, 2006). Similar to studies where endoglucanases were added at optimum levels in different bread making processes and resulted in reduced crumb firmness (Harada *et al.*, 2000; Harada *et al.*, 2005) the commercial and pure endoglucanases significantly ( $P<0.05$ ) increased softness at different concentrations in the present study.

#### *Baking trial 4*

In order to see if these enzymes can work synergistically when combined, baking trial 4 was conducted. In baking trial 4, the commercial endoxylanase and endoglucanase were combined at different concentrations, but when both were combined at medium concentrations it resulted in a very sticky dough. The pure endoxylanase could be added at a medium concentration with the pure endoglucanase at a low/medium concentration without influencing the dough characteristics. The pure endoxylanase contains only endoxylanase, while the commercial endoxylanase contains endoxylanase as well as  $\alpha$ -amylase. The commercial endoglucanase contains endoglucanase, endoxylanase,  $\alpha$ -amylase and cellobiohydrolase, while the pure endoglucanase contains only endoglucanase and endoxylanase activity. When these commercial enzymes were combined they could only be used at the lowest concentrations, otherwise they had a negative influence on dough characteristics. When combining the pure enzymes the dough characteristics were not altered because the pure enzymes contained fewer enzymes than the commercial enzymes.

No significant ( $P>0.05$ ) differences in bread weight were obtained. Three different enzyme combinations caused a significant ( $P<0.05$ ) increase in bread height (**Figs. 3.13 & 3.14**). The commercial endoxylanase added at a medium concentration with the commercial endoglucanase at a low concentration significantly ( $P<0.05$ ) increased bread height.

The endoxylanase increased loaf height, but when used in combination with endoglucanase an additional increase can be observed. Endoxylanases are known to increase bread height (Hammer, 1995; Poutanen, 1997; Courtin *et al.*, 1999, 2001; Hille & Schooneveld-Bergmans, 2004; Hille, 2005;) and when used in combination with endoglucanases an additional increase in loaf height can be expected (Dr. J.D.R Hille, Product Development Manager, DSM Baking Enzymes, Delft, the

Netherlands, personal communication, 2008). Cellobiohydrolases, endoglucanases and  $\beta$ -glucosidases work together to break down the cellulose polymer (Aristidou & Penttilä, 2000). Cellulases and endoxylanases have been shown to work synergistically as the hydrolysis of cellulose enables endoxylanase to hydrolyse arabinoxylans which are partly intertwined with the cellulose (Hille, 2005). This will result in a moderate release of water bound to the arabinoxylans which is then redistributed among other components of the dough. This results in improved extensibility of the gluten, improved gas retention, reduced resistance to rise, increased loaf volume and a softer crumb. From the results of baking trial 4 it seems as if the commercial enzymes produce a slightly higher bread height, although not significant ( $P>0.05$ ).

From the results of baking trial 4, tendencies could be seen; all the combinations of the pure endoxylanase and pure endoglucanase seem to decrease the number of holes, area of the holes and the volume of the holes (**Figs. 3.15-3.17**). These results were, however, not significant ( $P>0.05$ ). If these results were significant it would produce in a finer crumb texture. Compared to the control, the slice brightness was also not significantly ( $P>0.05$ ) increased. It seems that when the endoglucanases are combined with the endoxylanases the slice brightness is affected in such a way that an increase in slice brightness is not observed. The use of endoxylanases may result in slightly bigger or deeper cells or holes and a coarser crumb texture with more shadows which results in a decrease in overall slice brightness. When the enzymes were combined in baking trial 4, no significant ( $P>0.05$ ) increases in softness were seen on day 1. The enzymes did not work synergistically in increasing the softness of the bread crumb. It may be possible that the enzyme combinations did not result in an increase in crumb softness on day 1, but will lead to an increase in crumb softness over time. During a study were endoxylanases from fungal and bacterial origin were combined with cellulase a significant increase in softness was observed on day 4 and day 7, but this was not observed on day 1 after baking (Hille, 2005). It is known that  $\alpha$ -amylases retard bread staling (Miller *et al.*, 1953; Bowles, 1996; Linko *et al.*, 1997; Poutanen, 1997; Gray & Bemiller, 2003; Goesaert *et al.*, 2005; Caballero *et al.*, 2006). It might be possible that the level of  $\alpha$ -amylase used in the formulation was not the optimum level for retarding bread staling as it is suggested by the supplier to use 2-25 ppm  $\alpha$ -amylases per flour weight. The level of  $\alpha$ -amylase was subsequently decreased to be able to see the effect of the other enzyme on dough and bread quality characteristics. The crumb softness might be increased when the level of  $\alpha$ -amylase is increased.

## Conclusion

Both commercial and pure endoxylanases resulted in increased bread height and crumb softness, though with the pure endoxylanase these results were obtained at lower levels. In addition the pure

endoxyylanase resulted in increased slice brightness. Both commercial and pure endoglucanases resulted in increased slice brightness, irrespective of level of enzymes used. An increase in crumb softness was, however, only observed at the lowest concentrations of these enzymes. Pure enzymes could thus be beneficial to use as soon as it becomes more available and cost-effective. The use of individual, pure enzymes could be desirable in future as specific bread quality characteristics could be targeted to be improved.

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## **CHAPTER 4**

**The effect of commercial and pure endoxylanases, endoglucanases and their combination on wheat flour bread quality tested on commercial scale**



## **The effect of commercial and pure endoxylanases, endoglucanases and their combination on wheat flour bread quality tested on commercial scale**

### **Abstract**

Baking trials were conducted on commercial scale to evaluate commercial and pure endoxylanases and endoglucanases for their ability to improve wheat flour bread quality. A commercial endoxylanase (GH family 11) from *Aspergillus niger* and a commercial endoglucanase from *Trichoderma reesei* were evaluated together with a pure endoxylanase (GH family 11) and pure endoglucanase (GH family 7) from *T. reesei*. The effect of these enzymes on dough quality, bread weight, bread height, bread volume and softness of crumb was determined. In addition a digital imaging system (C Cell) was used to evaluate bread slice characteristics and overall crumb texture. Proof height was significantly ( $P<0.05$ ) increased by the pure endoxylanase and the commercial endoglucanase as well as one enzyme combination. Loaf height was significantly ( $P<0.05$ ) increased by the pure endoxylanase, the pure endoglucanase and one enzyme combination. The bread volume was significantly ( $P<0.05$ ) increased by all the individual enzymes being tested. Three enzyme combinations also increased bread volume significantly ( $P<0.05$ ). The number of cells was significantly ( $P<0.05$ ) decreased by the commercial endoxylanase, the pure endoxylanase and one enzyme combination. The area of the cells was significantly ( $P<0.05$ ) increased by the pure endoxylanase and one enzyme combination. The cell volume was significantly ( $P<0.05$ ) increased by the pure endoxylanase, the pure endoglucanase and two enzyme combinations. Slice brightness was significantly ( $P<0.05$ ) decreased by the commercial endoxylanase, the pure endoxylanase as well as the pure endoglucanase. Two commercial enzyme combinations significantly ( $P<0.05$ ) decreased the slice brightness. The crumb softness was not affected by any of the individual enzymes or enzyme combinations on day 1 or day 4.

### **Introduction**

In the Chorleywood Bread Process (CBP), all the ingredients are mixed in an ultra-high speed mixer for a few minutes; this process can also be described as a “no-time” dough bread making process which uses mechanical development (Mondal & Datta, 2008). The bread making process mainly used in South-Africa is derived from the CBP and can be described as a “no-time” straight dough process (Arie Wessels, Sasko Strategic Services, Paarl, South Africa, personal communication, 2008). This method employs a short dough development time in which all ingredients are incorporated in the initial mixing step. There is a reduction in total processing time due to the relative short fermentation time (Cauvain & Young, 2006). Space savings is an additional advantage since there is no need to keep dough at the different stages of development and

fermentation as would be required for bulk fermentation. The disadvantage of this method is the need for larger quantities of refrigerated water to control final dough temperature during mixing. This process can be used to evaluate untreated bread-wheat flour quality as well as a variety of bread making ingredients. Generally, enzymes are used to improve dough strength and elasticity; ensure a uniform and improved crumb structure; improve bread volume; and to increase shelf life (Terrazas, 2008). Enzymes most widely being used in the baking industry are: fungal  $\alpha$ -amylases; endoxylanases; lipolytic enzymes; glucose oxidases; proteases; intermediate heat stable  $\alpha$ -amylases and glucoamylases (Popper, 2008).

Most research reported until now usually employed individual wheat cultivars in small-scale baking trials (Harada *et al.*, 2000; Harada *et al.*, 2005; Jiang *et al.*, 2005; Caballero *et al.*, 2006; Collins *et al.*, 2006; Shah *et al.*, 2006; Dornez, 2007). Wheat flour used for bread making in South Africa is, however, usually a blend of different wheat cultivars. In addition, when conducting small-scale baking trials, a straight dough method is generally used. The formulation, processing conditions as well as equipment used is also different to that of commercial baking processes. Evaluating the effect of enzymes on a commercial scale, using a leaner formulation would be advantageous. The latter formulation would allow the effects of the enzymes to be observed more prominently.

The objectives of this study were therefore to:

- evaluate the effect of commercial and pure endoxylanases and endoglucanases on dough and bread quality characteristics such as bread weight, bread height, bread volume, crumb texture, slice brightness and crumb softness on a commercial scale; and
- evaluate the effect of a combination of the respective enzymes on dough and bread quality characteristics (bread weight, bread height, bread volume, crumb texture, slice brightness and crumb softness) on a commercial scale.

## **Materials and methods**

### *Flour, enzymes, ingredients and chemicals*

Commercial white bread flour supplied by Pioneer Foods trading as Sasko (Paarl, South Africa) was used. The enzymes, ingredients and chemicals used were as described in Chapter 3.

### *Determination of moisture, ash and protein contents*

Moisture, ash and protein contents were measured as described in Chapter 3.

*Rheological measurements*

Consistograph, farinograph, avleograph, mixograph as well as falling number tests were conducted as described in Chapter 3. All the rheological measurements were only conducted once.

*Enzyme activity measurements*

The enzyme activities were measured as described in Chapter 3.

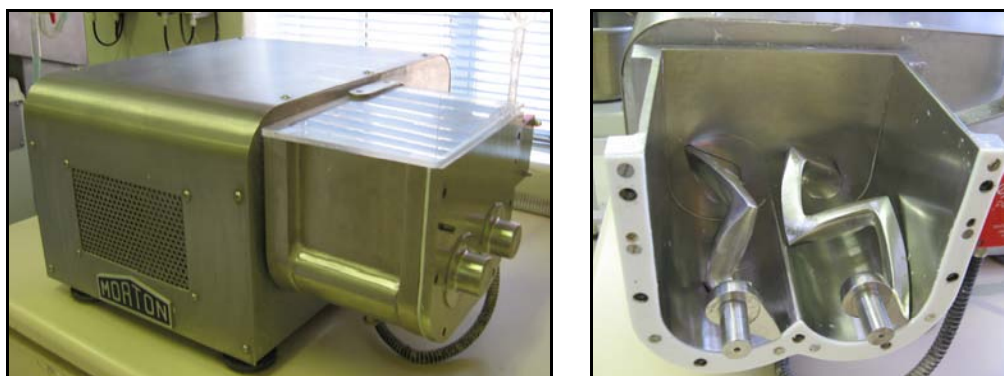
*Bread making process*

A “no-time” straight dough process was used with the basic formulation detailed in **Table 4.1**. The ingredients comprised fresh compressed yeast, edible salt and edible sugar, shortening, soyflour, chemically pure ascorbic acid and  $\alpha$ -amylase. The water absorption was optimised for the control sample (based on the moisture and protein contents of the flour) and kept constant for all enzyme treatments. The ascorbic acid and all the enzymes were made up to pre-determined concentrations using distilled water. The dry ingredients were mixed for 2 min to ensure even distribution of all the ingredients. The dough was optimally mixed until dough development, in a Z-blade high speed mixer (No. 0 Duplex Mixer, Morton Machine Company Ltd., Scotland, UK) (**Fig. 4.1**). The Mixograph peak time was correlated with the dough development times as estimated by an experienced baker who determined the appropriate mixing times. The average mixing time of the control samples was then taken as the standard mixing time which was kept constant for all enzyme treatments. The dough temperature was recorded with a digital thermometer (HI 146-00, Hanna Instruments, South Africa) approximately 1 min after mixing. The dough was covered with a plastic dome and allowed to rest for 5 min. The dough was divided into two equal sized portions and then scaled to 890 g to yield a final bread mass of 800 g (after 1 hr cooling at ambient temperature). The dough was rounded by hand, the dough consistency recorded and the dough covered with a plastic dome and allow to proof at ambient temperature for 5 min. The dough was passed through a moulder (Mini Status Moulder 350, Macadams, South Africa) (**Fig. 4.2**), placed seam side down in the bread pans (268 x 100 x 114 mm) and proofed in a fermentation cabinet (Proover, Macadams, South Africa) for 52 min at 40°C and 80% relative humidity (RH). After proofing (fermentation) the height of the respective loaves were recorded in mm and subsequently baked in a pre-heated convection oven (Convecta 8, Macadams, South Africa) for 26 min at 200°C. The loaves were removed from the pans after baking and subsequently weighed. The loaves were left to cool for 1 hr at room temperature. The height was measured with a graduated height meter (**Fig. 4.3**) (Peter Rassloff Instruments and Services Pty Ltd., Cape Town) and bread volume was measured with a bread loaf volume meter (**Fig. 4.4**). Loaves were placed in plastic bags (Proton Packaging, Paarl) and stored for 24 h at 22°C when quality evaluations (crumb texture and slice brightness) were

performed. The results for proof height, bread weight, bread height and bread volume are the average taken for the two loafs of each treatment. Crumb softness was performed on the one loaf on day 1 and on the other loaf, 4 days after baking.

**Table 4.1** Details of the bread making formulation

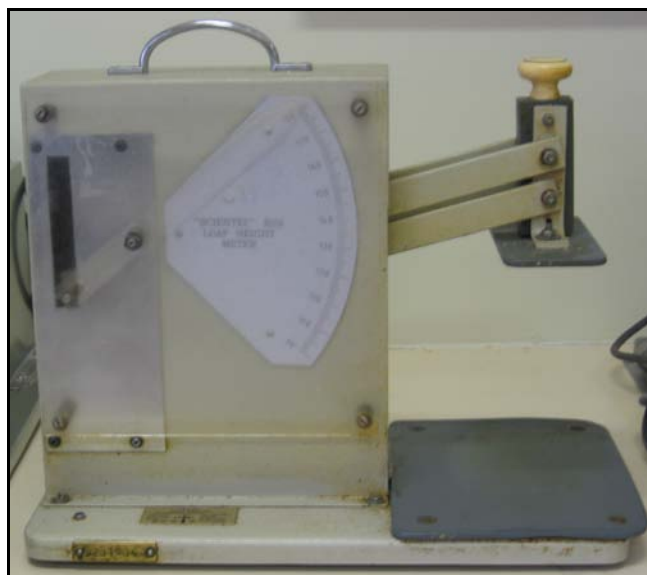
| Ingredients                   | Formulation |
|-------------------------------|-------------|
| Flour (14% m.b), %            | 1400        |
| Distilled water               | Optimum     |
| Fresh compressed yeast, %     | 2.6         |
| Salt (NaCl), %                | 2           |
| Sugar, %                      | 1           |
| Shortening (fat), %           | 0.25        |
| Soyflour, %                   | 0.20        |
| Ascorbic acid, ppm            | 80          |
| Fungal $\alpha$ -amylase, ppm | 7           |



**Figure 4.1** The Z-blade mixer used for mixing dough until dough development.



**Figure 4.2** The moulder used for moulding the dough.



**Figure 4.3** The graduated height meter used for measuring loaf height (in mm).



**Figure 4.4** The bread loaf meter used for determining loaf volume (in mL).

#### *Baking trials for enzyme evaluation*

One commercial baking trial was conducted. The formulation used is given in **Table 4.1**. From the results of the 100 g baking trials the lowest and highest concentration of each enzyme was used in the commercial baking trial. These included the commercial endoxylanase (5 & 20 ppm), the pure endoxylanase (10 & 40 ppm), the commercial endoglucanase (3 & 8 ppm) and the pure endoglucanase (40 & 150 ppm). The ascorbic acid and all the enzymes were made up to pre-determined concentrations using distilled water. New stock solutions were made daily. The commercial and pure endoxylanases and endoglucanases were evaluated in combination as

illustrated in (**Table 4.2**). When enzymes are combined lower levels of each individual enzyme can be used. Therefore the low and medium concentrations of each enzyme (from the 100 g baking trials) were used.

**Table 4.2** The concentrations of the individual enzymes and the enzyme combinations

| Enzymes       | Concentration (ppm)     |                          |                   |                    |
|---------------|-------------------------|--------------------------|-------------------|--------------------|
|               | Commercial endoxylanase | Commercial endoglucanase | Pure endoxylanase | Pure endoglucanase |
| Combination 1 | 5                       | 5                        | -                 | -                  |
| Combination 2 | 12                      | 3                        | -                 | -                  |
| Combination 3 | 12                      | 5                        | -                 | -                  |
| Combination 4 | -                       | -                        | 10                | 80                 |
| Combination 5 | -                       | -                        | 24                | 40                 |
| Combination 6 | -                       | -                        | 24                | 80                 |

#### *Evaluation of dough and bread quality*

The overall characteristics of the dough were visually evaluated and compared to those of the control sample after resting. These included elasticity, surface texture and general handling and moulding of the dough. Any deviations from the control sample were recorded. Physicochemical characteristics of the bread loaves including weight (g), height (mm), volume (mL) and crumb texture were assessed. The results for proof height, bread weight, bread height and bread volume are the average taken for the two loafs of each treatment. The texture analysis of the bread was performed after cooling and 24 h of storage at 22°C. The loaves were cut using an automatic bread slicer (Model J, Kühn Machines, South Africa). The analysis was performed on 6 respective slices obtained from each loaf of bread. Crumb grain characteristics were assessed using a C Cell digital image analysis system (Colibre Control International, Appleton, UK). The crumb grain characteristics recorded were: total slice area (pixels), maximum height (pixels), height:width ratio, number, area and volume of cells and holes; and slice brightness (pixels). Immediately after digital imaging the same slices were used to measure crumb softness. This was done using a Zwick material testing machine (Model Z005, Wirsam, Germany) equipped with a 35 mm diameter aluminium cylindrical probe at a cross head speed of 100 mm/s. Bread slices (25 mm total height) were compressed to a depth of 40%. Crumb softness was performed on the one loaf on day 1 and on the other loaf on day 4.

#### *Experimental design*

A full-factorial experimental design, performed with four factors at three levels, was used.

### Statistical analysis

Statistical analysis was performed and graphs compiled using STATISTICA version 8 (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was performed to compare average measurements between samples to determine absolute differences. Analysis of covariance (ANCOVA) was used to compensate for the effect of dough temperature. Pearson correlations were calculated to determine relationships between continuous measurements. The bar around the average represents the 95% confidence interval for the average measurements. The Fisher least significance (LSD) *post-hoc* testing was used. All references to statistical differences indicate significant statistical differences at  $P < 0.05$ .

## Results

### *Quality attributes of commercial white bread flour*

A new batch of flour was used in conducting the commercial baking trial and the quality attributes of the flour were determined to ensure that the flour used was as consistent as possible (**Table 4.3**).

**Table 4.3** Quality attributes of commercial white bread flour

| <b>Quality attribute</b>                        |       |
|---|-------|
| Moisture, %                                     | 14.7  |
| Ash, % (dry weight)                             | 0.80  |
| Protein, % (12% mb)                             | 10.8  |
| <u>Consistograph</u>                            |       |
| Water absorption, % (14% mb)                    | 62.4  |
| Tolerance                                       | 276   |
| <u>Alveograph</u>                               |       |
| Tenacity (P), mm                                | 81    |
| Distensibility (L), mm                          | 89    |
| Deformation energy (W), $\times 10^{-4}$ Joules | 226   |
| Curve configuration ratio (P/L)                 | 0.92  |
| <u>Farinograph</u>                              |       |
| Water absorption, % (14% m.b.)                  | 59.6  |
| Dough development time, min                     | 7.0   |
| Stability, min                                  | 9.2   |
| <u>Mixograph</u>                                |       |
| Water absorption, %                             | 59.45 |
| Peak time, min                                  | 3.64  |
| <u>Falling number</u>                           |       |
| Falling number, s                               | 401   |



### *Enzyme activity measurements*

The activities of the enzymes were measured against different substrates before the trials commenced and is summarised in **Table 3.5 (Chapter 3)**.

### *Evaluation of dough and bread quality*

#### Dough quality

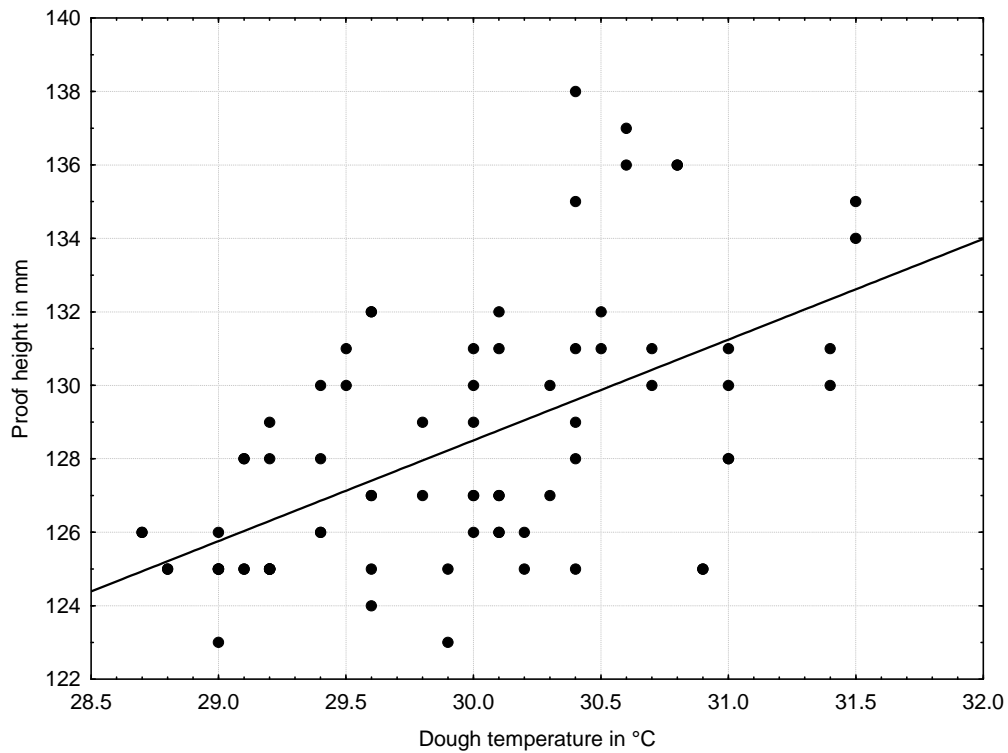
The pure endoxylanase used at 10 and 40 ppm resulted in sticky doughs as well as the commercial endoglucanase at 3 and 8 ppm. When 5 ppm commercial endoxylanase was added with 5 ppm endoglucanase a sticky dough was obtained as well as with 12 ppm commercial endoxylanase added with 3 ppm endoglucanase. When the pure endoxylanase was added at 24 ppm with the pure endoglucanase at 40 ppm it also resulted in a sticky dough.

#### Bread weight

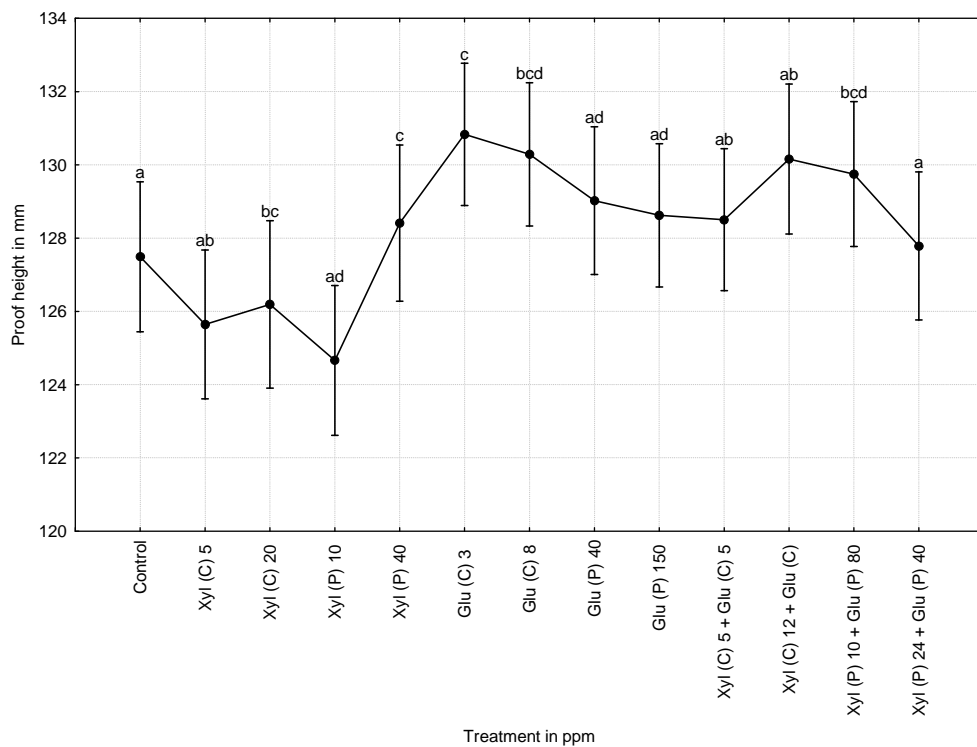
No significant ( $P>0.05$ ) differences were observed for the average bread weights (results not shown).

#### Proof height

The average dough temperatures were compared to the average proof heights (**Fig. 4.5**) and the correlation was found to be significant ( $r=0.57$ ,  $P<0.05$ ). This indicated higher dough temperatures resulted in higher proof heights. Therefore, the effect of the dough temperature was removed when the statistical analysis was performed. When the pure endoxylanase was added at 40 ppm it resulted in a significant ( $P<0.05$ ) increase in proof height (**Fig. 4.6**). The commercial endoglucanase also significantly increased proof height at 3 and 8 ppm. One enzyme combination significantly increased proof height, i.e. the pure endoxylanase at 10 ppm added with the pure endoglucanase at 80 ppm.



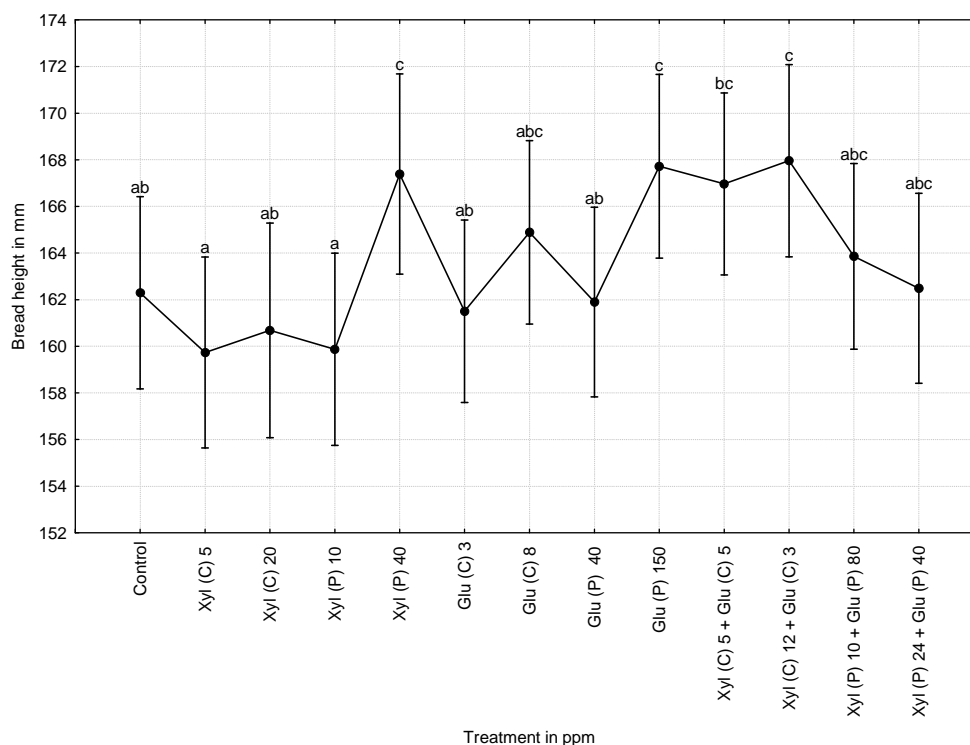
**Figure 4.5** Correlation between the dough temperatures and the average proof heights ( $r=0.57$ ;  $P<0.01$ ).



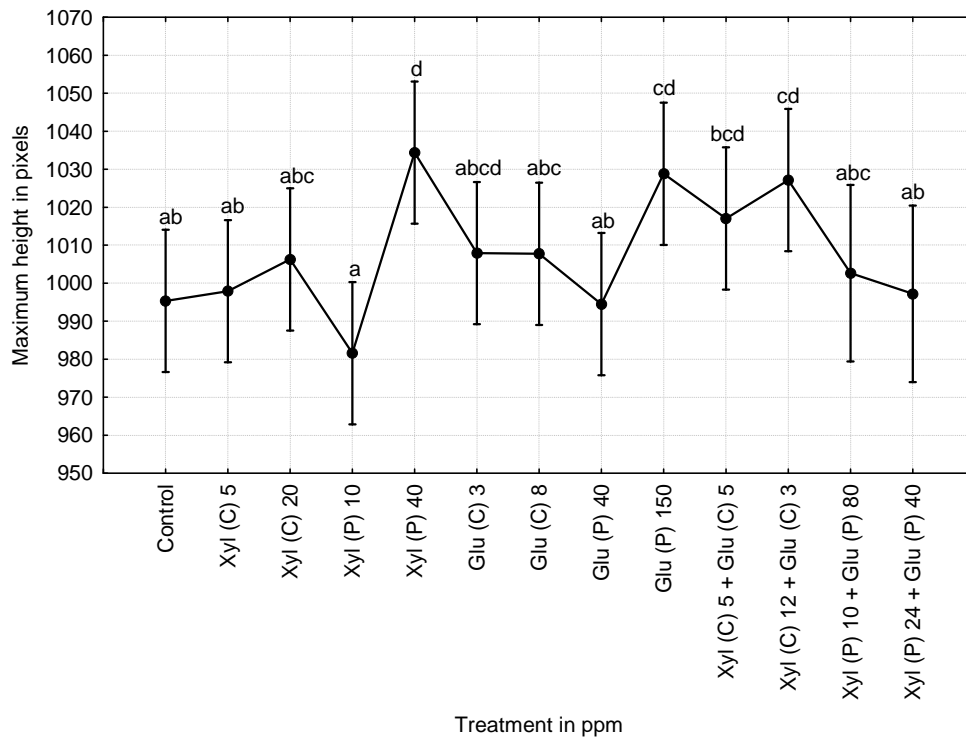
**Figure 4.6** Differences between the average proof heights, measured manually, of the doughs determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

### Bread height

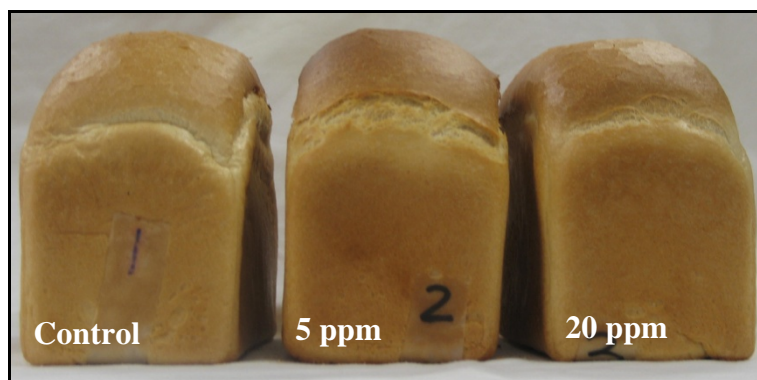
The average dough temperatures were compared to the average bread heights and the correlation was not significant ( $r=-0.05$ ,  $P>0.05$ ) (results not shown). Higher dough temperatures did not result in higher bread heights; however, the effect of the dough temperature was removed when the statistical analysis was performed. From the manual measurements (**Fig. 4.7**) significant ( $P<0.05$ ) increases in bread height were observed. These include the pure endoxylanase at 40 ppm, the pure endoglucanase at 150 ppm and the commercial endoxylanase at 12 ppm added with the commercial endoglucanase at 3 ppm. From the C Cell measurements (**Fig. 4.8**) the same results were obtained; the pure endoxylanase at 40 ppm, the pure endoglucanase at 150 ppm as well as the commercial endoxylanase at 12 ppm added with the commercial endoglucanase at 3 ppm significantly ( $P<0.05$ ) increased bread height. **Figures 4.9** and **4.10** shows examples of loafs were individual enzymes as well as enzymes combinations are compared to the control. **Figure 4.11** shows digital images obtained from the C Cell to measure bread height.



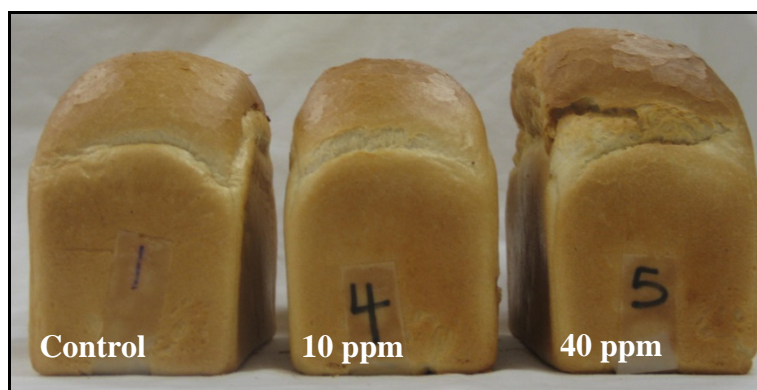
**Figure 4.7** Differences between the average bread heights (manually) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



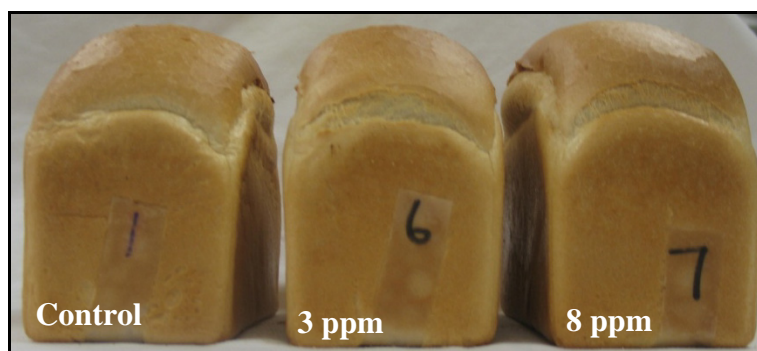
**Figure 4.8** Differences between the average bread heights (digital imaging) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



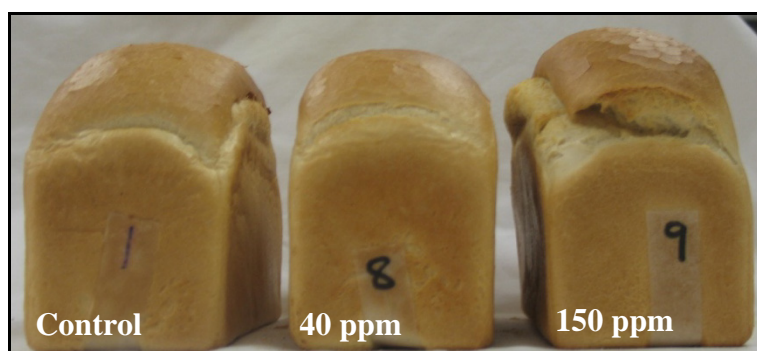
**A**



**B**

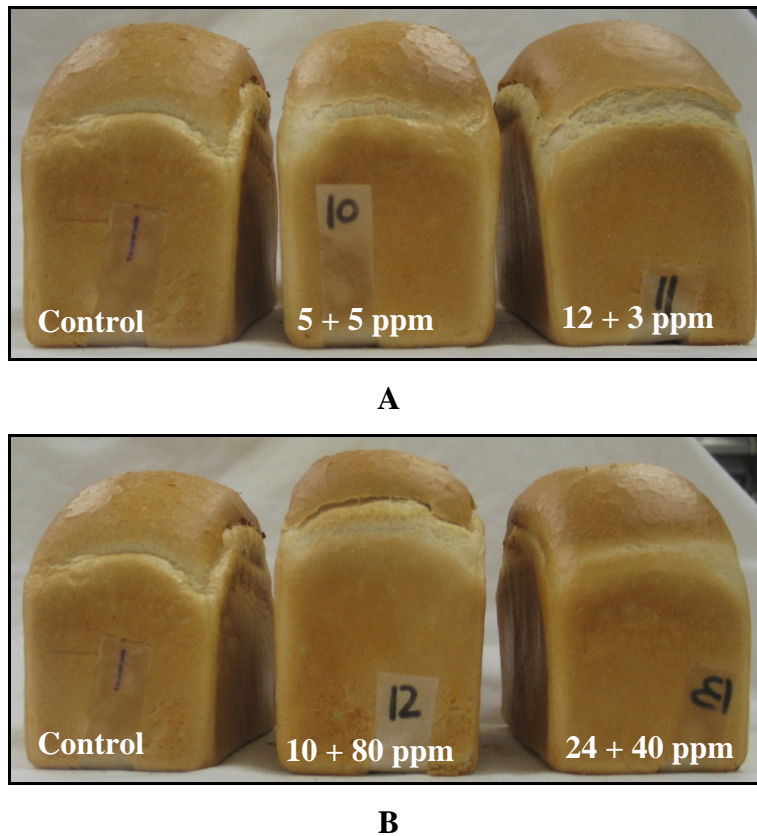


**C**

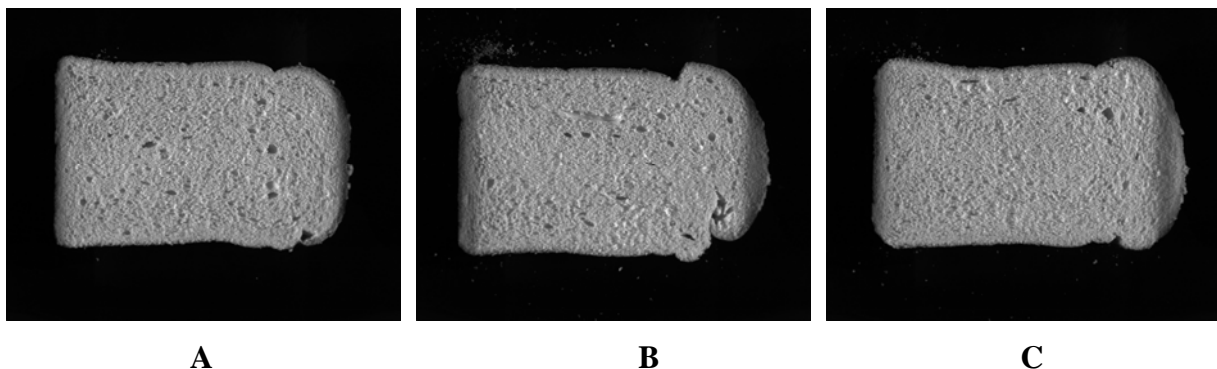


**D**

**Figure 4.9** Control compared to the commercial endoxylanase (A), control compared to the pure endoxylanase (B), control compared to the commercial endoglucanase (C) and control compared to the pure endoglucanase (D).



**Figure 4.10** Control compared to the commercial enzyme combinations (A) and the control compared to the pure enzyme combinations (B).

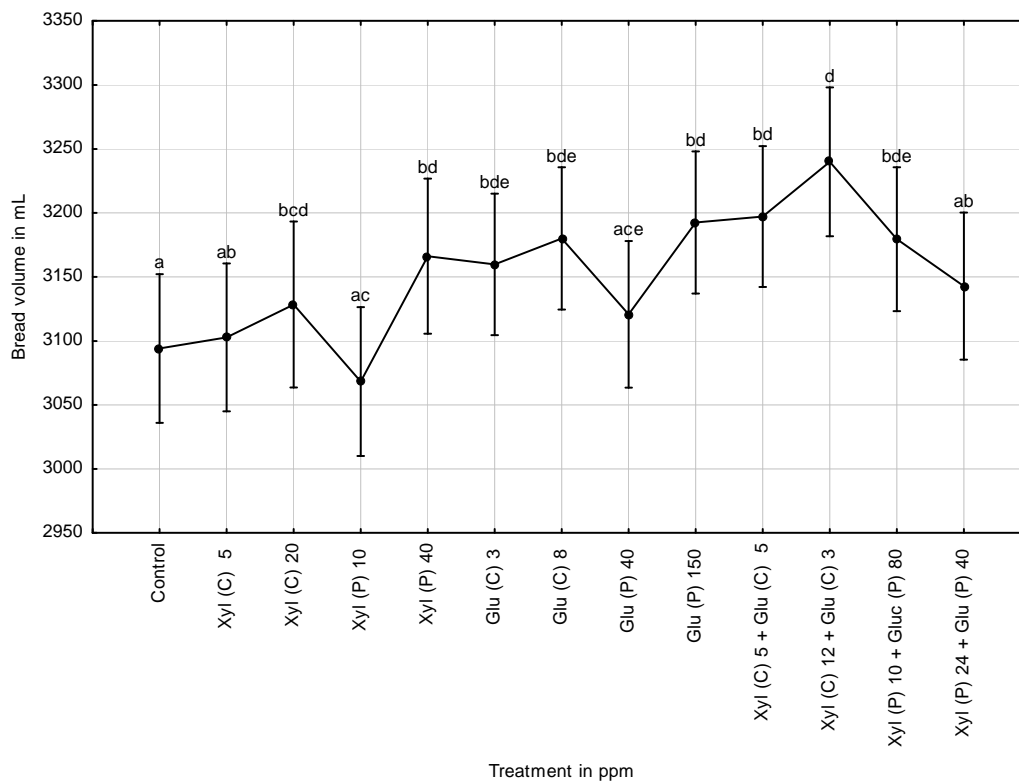


**Figure 4.11** Digital images obtained from the C Cell were the control (A) is compared to the pure endoglucanase at 150 ppm (B) and the pure endoxylanase at 40 ppm (C).

#### Bread volume

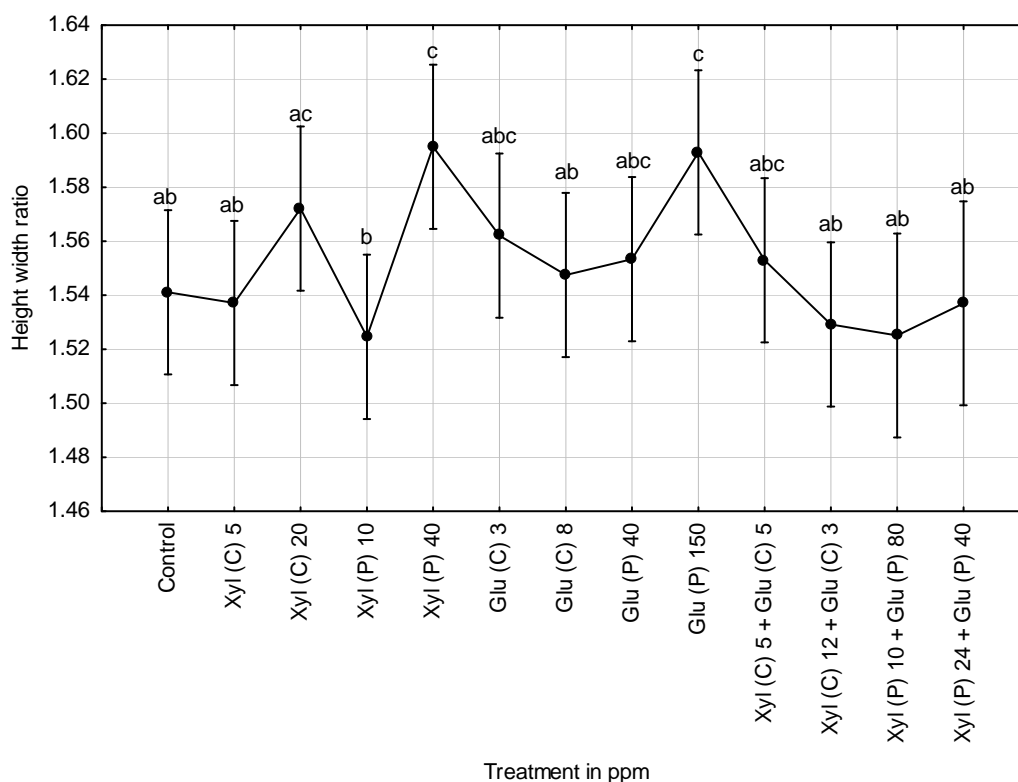
The average dough temperatures were compared to the average bread volumes and the correlation was not significant ( $r=0.18$ ,  $P>0.05$ ) (results not shown). Higher dough temperatures did not result in increased bread volumes; however, the effect of the dough temperatures was removed when the statistical analysis was performed. From the manual measurements (**Fig. 4.12**) the bread volume was significantly ( $P<0.05$ ) increased by the following individual enzymes: commercial endoxylanase at 20 ppm, pure endoxylanase at 40 ppm, the commercial endoglucanase at 3 and 8 ppm, the pure endoglucanase at 150 ppm. The following enzyme combinations also increased bread

volume significantly ( $P<0.05$ ): commercial endoxylanase at 5 ppm added with the commercial endoglucanase at 5 ppm, the commercial endoxylanase at 12 ppm added with the commercial endoglucanase at 3 ppm, as well as the pure endoxylanase at 10 ppm added with the pure endoglucanase at 80 ppm. The C Cell measurements (**Fig. 4.13**) indicated that only the pure endoxylanase at 40 ppm and the pure endoglucanase at 150 ppm significantly ( $P<0.05$ ) increased bread volume.



**Figure 4.12** Differences between the average bread volume (manually) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



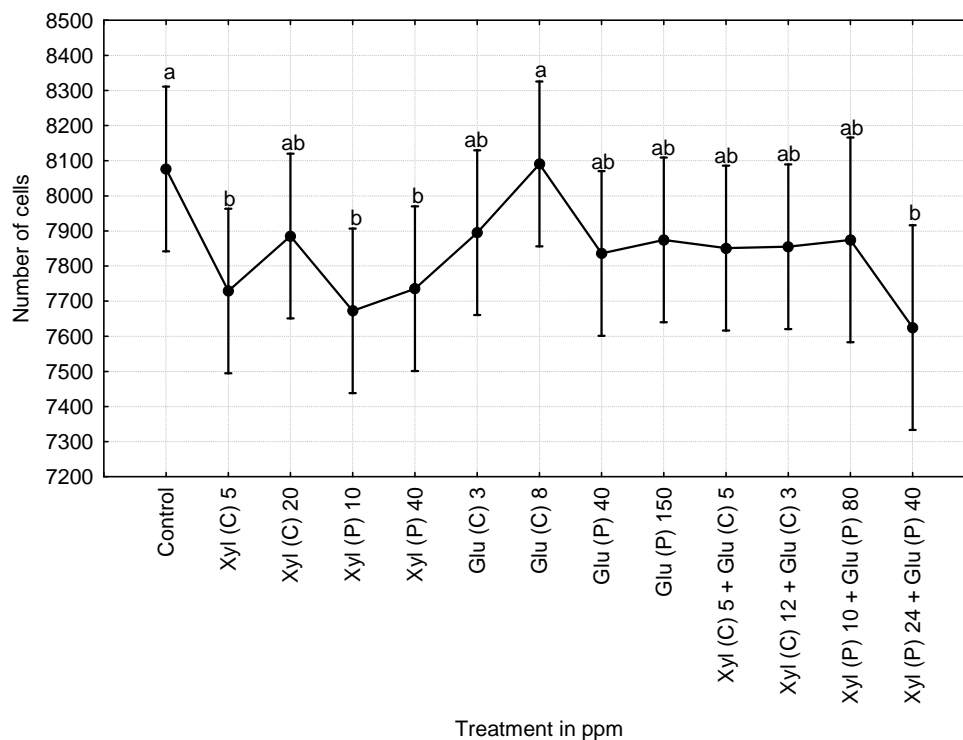


**Figure 4.13** Differences between the average bread volume (digital imaging) determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

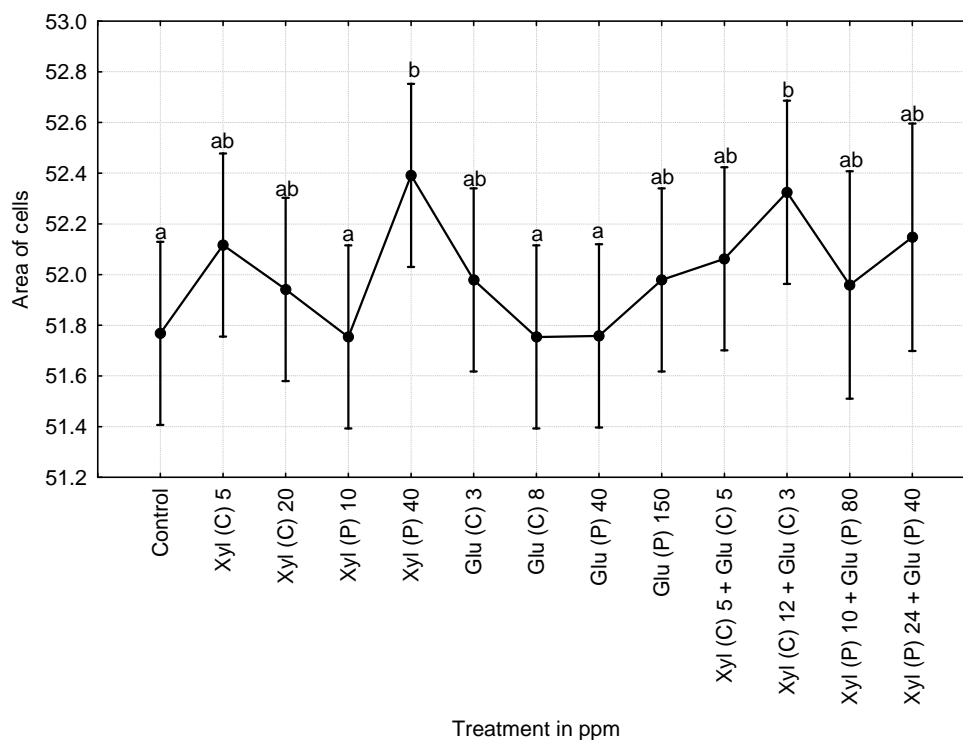
#### Bread crumb texture

The number of the cells (**Fig. 4.14**) was significantly ( $P<0.05$ ) decreased by the commercial endoxylanase at 5 ppm, the pure endoxylanase at 10 and 40 ppm and the pure endoxylanase at 24 ppm added with the pure endoglucanase at 40 ppm. The area of the cells (**Fig. 4.15**) was significantly ( $P<0.05$ ) increased by the pure endoxylanase at 40 ppm and the commercial endoxylanase at 12 ppm added with the commercial endoglucanase at 3 ppm. The cell volume (**Fig. 4.16**) was significantly ( $P<0.05$ ) increased by the pure endoxylanase at 40 ppm, the pure endoglucanase at 150 ppm, the commercial endoxylanase at 12 ppm added with the commercial endoglucanase at 3 ppm as well as the pure endoxylanase at 24 ppm added with the pure endoglucanase at 40 ppm.

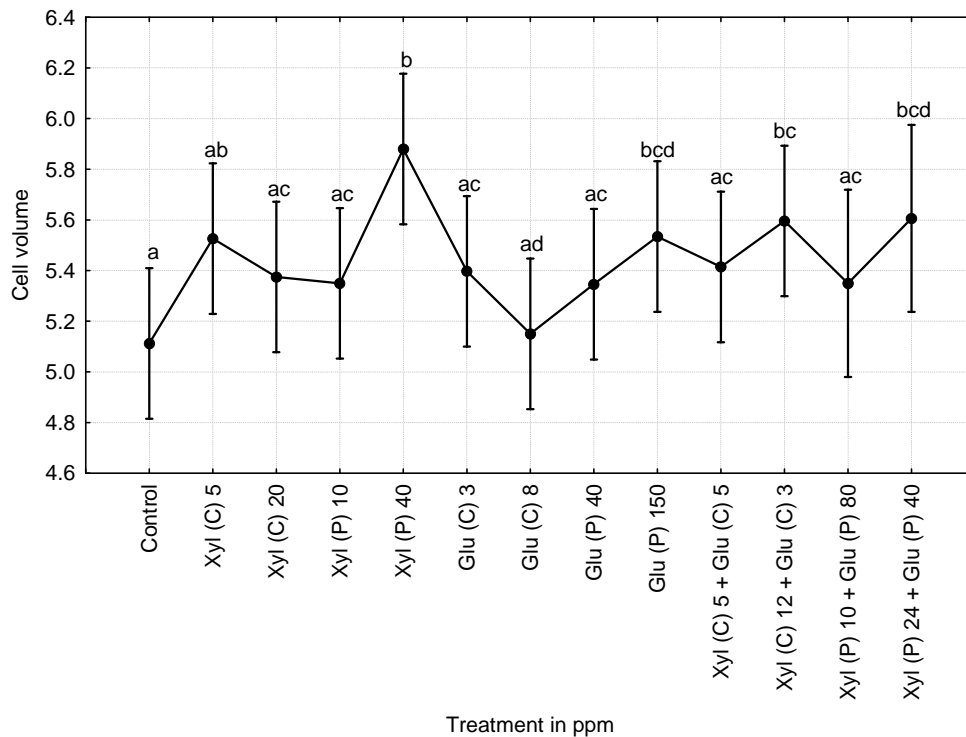




**Figure 4.14** Differences between the average number of cells determined by analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 4.15** Differences between the average area of the cells determined by analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



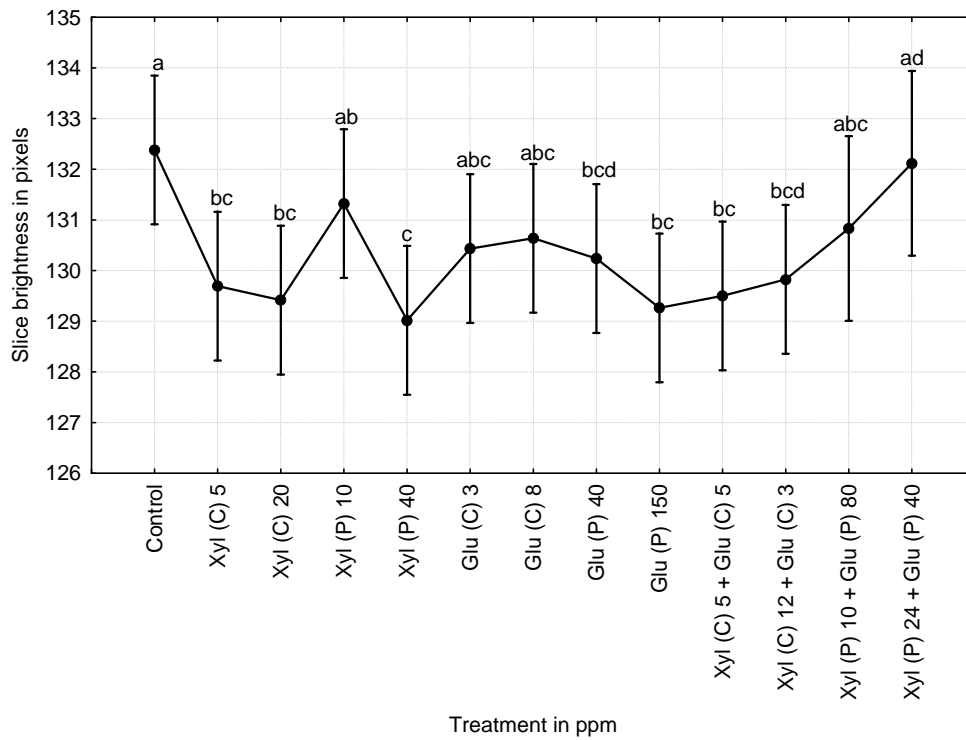
**Figure 4.16** Differences between the average volume of the cells determined by analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

#### Slice brightness

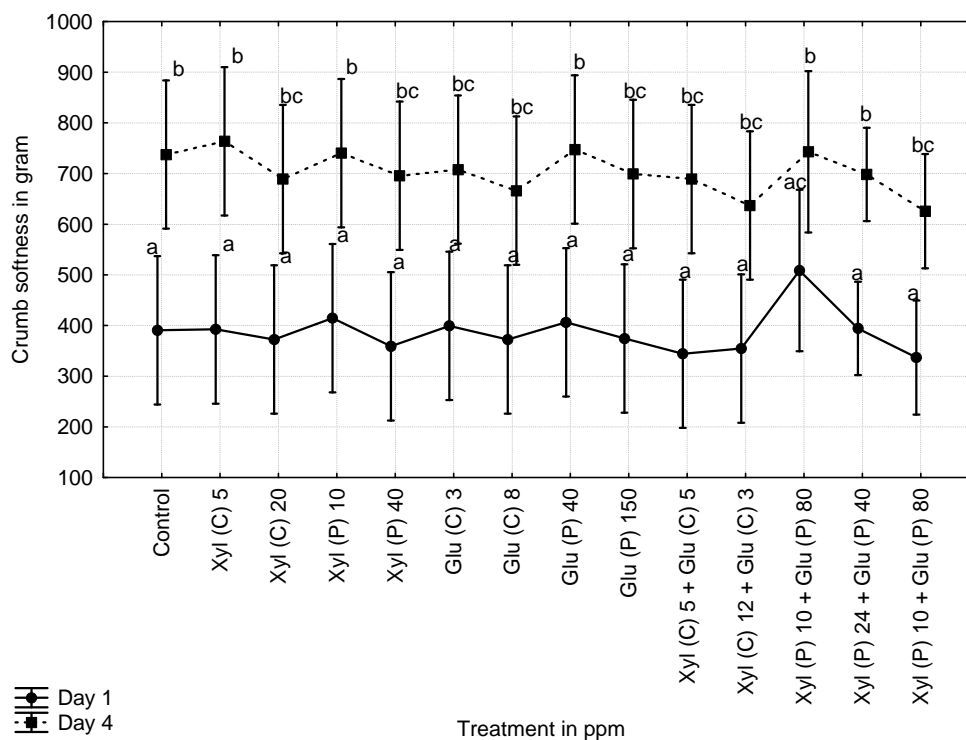
The slice brightness (**Fig. 4.17**) was significantly ( $P < 0.05$ ) decreased by the following enzymes treatments: the commercial endoxylanase at 5 ppm and 20 ppm, the pure endoxylanase at 40 ppm as well as the pure endoglucanase at 150 ppm. When the commercial endoxylanase at 5 ppm was added with the commercial endoglucanase at 5 ppm the slice brightness was significantly ( $P < 0.05$ ) decreased. This was also observed when the commercial endoxylanase at 12 ppm was added with the commercial endoglucanase at 3 ppm.

#### Crumb softness

The crumb softness (**Fig. 4.18**) of the loaves were not significantly ( $P > 0.05$ ) affected by any of the individual enzyme treatments or the enzyme combinations on day 1 or on day 4.



**Figure 4.17** Differences between the average slice brightness determined by analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).



**Figure 4.18** Differences between the average crumb softness, measured on day 1 and on day 4, determined with analysis of variance (ANOVA). Error bars denote 0.95 confidence intervals. Different letters indicate significant differences obtained from LSD analysis (Xyl=endoxylanase; Glu=endoglucanase; C=commercial; P=pure).

## **Discussion**

### Dough quality

The use of the pure endoxylanase, as well as a combination of the pure endoxylanase and endoglucanase, resulted in sticky doughs when the baking trial was conducted on commercial scale. This was not observed when the 100 g baking trials were performed and could be a result of the mixer used. A high speed mixer was used in conducting the baking trial and due to the high energy input of the mixer the dough temperatures increased rapidly. As the mixer heats up and the room temperature rises (due to the high temperatures in the summer) the temperature of the dough would further increase. Therefore, the temperature of the water was strictly controlled in order to obtain consistent dough temperatures ( $30\pm1^{\circ}\text{C}$ ). Yet, slight fluctuations in the dough temperatures occurred which was taken into account when the statistical analyses were performed. In the commercial baking trial, the commercial endoglucanase, as well as both the commercial enzyme combinations resulted in sticky doughs. The concentrations used for the endoglucanase in this baking trial were the same as those used in the small-scale baking trials, but during the small-scale baking trials it did not result in sticky doughs. Only the addition of medium concentrations of both the commercial endoxylanase and endoglucanase resulted in sticky doughs in the small-scale baking trials. The fact that lower concentrations of the commercial endoglucanase and the commercial enzyme combinations resulted in sticky doughs when the baking trials were conducted on commercial scale can be attributed to the high energy input from the high speed mixer used. From the enzyme activity results (**Table 3.5; Chapter 3**) it is known the pure endoxylanase contained only endoxylanase activity, while the commercial endoxylanase contained endoxylanase and  $\alpha$ -amylase activities. The commercial endoglucanase contained endoglucanase, endoxylanase,  $\alpha$ -amylase and cellobiohydrolase activities, while the pure endoglucanase contained only endoglucanase and endoxylanase activities. The commercial enzymes were able to degrade more substrates than the pure enzymes and this could result in more water being released from the arabinoxylans (AX) as well as too much starch being degraded resulting in the formation of a sticky dough.

### Proof height

The results obtained for proof height were irrespective of the dough temperatures since the effect of the dough temperatures were removed when the statistical analysis was done. Several individual enzymes as well as one enzyme combination (**Fig. 4.6**) had the ability to increase proof heights when compared to the control. Improved bread heights and/or volumes were to be expected.

### Bread weight

There were no significant ( $P>0.05$ ) differences in bread weight between the treatments indicating the ingredients were accurately weighed and the dough was accurately divided and weighed. Differences in dough weight would influence bread height and volume. Therefore, ensuring consistent dough weight is extremely important especially when bread height and volume is measured. Inconsistencies could easily be incorrectly interpreted as the ability of the enzymes improving the height or volume of the bread.

### Bread height

The optimum temperatures of fungal  $\alpha$ -amylases are between 40 and 50°C (Hamer, 1991), endoxylanases have an optimum temperature between 40 and 50°C (Dekker & Richards, 1976) and endoglucanases between 50 and 80°C (Baldrain & Valaskova, 2008). These enzymes have higher optimum temperatures than the dough temperatures reached after mixing; therefore they possibly had a greater effect on bread height and volume during proofing and baking. These enzymes work more rapidly when exposed to the higher temperatures (during proofing and baking) after which they were inactivated. Therefore, the dough temperatures did not influence the bread height and volume. The effect of the dough temperatures was still removed when the statistical analysis was carried out. The pure endoxylanase and the pure endoglucanase significantly ( $P<0.05$ ) increased the loaf height (**Figs. 4.7 & 4.8**). According to the literature cited, both these enzymes have been shown to have a positive effect on loaf height (Hamer, 1991; Poutanen, 1997; Courtin *et al.*, 1999, 2001; Hille & Schooneveld-Bergmans, 2004; Hille, 2005; Harada *et al.*, 2000; Harada *et al.*, 2005). When the commercial endoxylanase was combined with the commercial endoglucanase, the bread height was also significantly ( $P<0.05$ ) increased (**Figs. 4.7 & 4.8**). It is known the addition of endoxylanases in combination with endoglucanases results in an additional increase in loaf height (Dr. J.D.R. Hille, Product Development Manager, DSM Baking Enzymes, Delft, the Netherlands, personal communication, 2008).

### Bread volume

Higher dough temperatures did not result in increased bread volumes as the temperatures reached after mixing were not near to the optimum temperatures required for the respective enzymes. From the manual measurements (**Fig. 4.12**) the bread volume was significantly ( $P<0.05$ ) increased by all the individual enzymes (at specific concentrations). The commercial endoxylanase combined with the commercial endoglucanase as well as the pure endoxylanase combined with the pure endoglucanase significantly ( $P<0.05$ ) increased bread volume. From the C Cell measurements (**Fig. 4.13**), it is apparent that only the pure endoxylanase as well as the pure endoglucanase increased

bread volume significantly ( $P<0.05$ ). The addition of small quantities of enzyme (in ppm) to the ingredients significantly ( $P<0.05$ ) increases the volume of the bread loaf, without requiring the addition of more of the other ingredients. The commercial endoxylanase derived from *A. niger* and the pure endoxylanase derived from *T. reesei* belongs to the GH family 11 which is known to have preference for water un-extractable arabinoxylans (WU-AX) (Hille, 2005). The WU-AX can hold up to ten times their weight in water (Kim & D'Appolonia, 1977). Therefore, the increase in bread volume can be attributed to the ability of the endoxylanases to hydrolyse the water un-extractable part of the arabinoxylans (AX). As a result of the hydrolysis, the previously bound water is released and redistributed to other components of the dough (damaged starch and gluten). This results in improved extensibility of the gluten, gas retention and reduced resistance to rise (Hille, 2005). The WU-AX also destabilises the dough structure and are present in fragments that can form physical barriers to the gluten network during dough development (Courtin & Delcour, 2002). The hydrolysis of WU-AX results in a more stable dough since the gluten will be able to hold more of the carbon dioxide released during yeast fermentation. This will result in an improved proof height, bread height and/or volume.

#### Bread crumb texture

The pure endoxylanase significantly ( $P<0.05$ ) decreased the number of cells (**Fig. 4.14**) and increased the area (**Fig. 4.15**) and volume (**Fig. 4.16**) of the cells. The commercial endoxylanase significantly ( $P<0.05$ ) decreased the number of cells whereas the pure endoglucanase significantly ( $P<0.05$ ) increased the cell volume. When combining the pure endoxylanase with the pure endoglucanase the number of cells were significantly ( $P<0.05$ ) decreased and the volume of the cells increased. The commercial endoxylanase combined with the commercial endoglucanase significantly ( $P<0.05$ ) increased the area and the volume of the cells. The use of the above mentioned enzymes or enzyme combinations (at the specific concentrations) significantly ( $P<0.05$ ) influenced one or more than one of the characteristics of the cells. Their use resulted in larger and/or deeper cells which led to a more open or coarser crumb texture.

#### Slice brightness

The following individual enzymes significantly ( $P<0.05$ ) decreased the slice brightness (**Fig. 4.17**): the commercial endoxylanase, the pure endoxylanase as well as the pure endoglucanase. Both the commercial endoxylanase and endoglucanase combinations significantly ( $P<0.05$ ) decreased the slice brightness. Most of these enzymes or enzyme combinations (which significantly ( $P<0.05$ ) decreased slice brightness) also had a significant ( $P<0.05$ ) effect on bread crumb texture. The bread crumb texture was influenced in such a way that it was more open and coarse which is attributed to

larger or deeper cells reflecting larger shadows. The large shadows would result in a decrease in the overall slice brightness.

### Crumb softness

None of the individual enzymes or the enzyme combinations significantly ( $P<0.05$ ) increased the crumb softness on day 1 or on day 4 (**Fig. 4.18**). During a study where endoxylanases from fungal and bacterial origin were combined with cellulases, a considerable increase in softness was observed on day 4 and day 7, but no visible difference was observed 1 day after baking (Hille, 2005). It might be possible that the enzyme concentrations used in this study was not the optimum concentrations required for increasing the shelf life of the bread.

### **Conclusion**

Similar results were obtained for both pure enzymes, i.e. bread height and loaf volume increased while slice brightness decreased. In addition, a more open and coarse crumb texture was observed for both pure enzymes. Loaf volume and bread height was not affected by the commercial enzymes. The more open crumb texture observed resulted in a decrease in slight brightness. When the commercial enzymes were added in combination the bread height was increased. Again the more open and coarse crumb texture resulted in a decreased slice brightness. Both the pure enzymes showed to be more effective compared to the commercial enzymes. It was possible to use a “no-time” straight dough process with a lean formulation to evaluate the effect of enzymes effectively. The benefit of this is that the effect of the enzymes is being tested in a process similar to that used in industry which would allow direct comparison.

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## **CHAPTER 5**

### **General discussion and conclusions**

## General discussion and conclusions

Endoxylanases are known to improve dough stability, oven spring, loaf volume, crumb structure and shelf life (Rouau *et al.*, 1994; Hamer, 1995; Poutanen, 1997; Popper, 1997; Courtin & Delcour, 2002; Hille & Schooneveld-Bergmans, 2004; Hille, 2005). Commercial cellulases (endoglucanases) can be used to increase bread loaf volumes, bread scores and reduced crumb firmness (Harada *et al.*, 2000; Harada *et al.*, 2005). It is known commercial enzymes do not exclusively contain only the specific enzyme indicated on the label, but also other enzymes that happened to be produced by the same source material/organism (Law, 2002). Therefore, commercial endoxylanase and endoglucanase were compared with their pure counterparts in terms of their ability to improve bread making quality. Before baking trials commenced, the activity of the enzymes was determined against different substrates to establish the concentrations to be used in the small (100 g) and commercial (700 g) scale baking trials.

The basic formulation (100 g) as described in AACC Approved method 10-10B, (AACC, 2000) made it difficult to observe the effects of the enzymes. Therefore, it was adjusted into a leaner formulation which allowed the effects of the enzymes to be more easily observed. From the results it was observed the commercial endoxylanase produced a slightly sticky, softer dough, while the pure endoxylanase produced a slightly dry, firmer dough. The effect of enzymes on dough characteristics is important as this will influence further processing; a dryer dough is more desirable and easier to handle. It has been noticed in industry endoxylanase tends to result in slightly sticky doughs.

Considering the average values for bread height, it was observed both the commercial and the pure endoxylanases were able to increase the bread height significantly compared to the control. However, to obtain the same effect, the pure endoxylanase could be used at a lower concentration (10 ppm) compared to the commercial endoxylanase (20 ppm).

The slice brightness was significantly ( $P < 0.05$ ) increased by the commercial and pure endoglucanases at all the concentrations used, as well as by the pure endoxylanase used at the highest concentration. It might be possible that the above mentioned enzymes decreased the area and/or volume of the cells which resulted in smaller cells, with smaller shadows. The latter led to an increase in slice brightness. It is known the use of endoglucanases will result in an improved crumb texture (Dr. J.D.R Hille, Product Development Manager, DSM Baking Enzymes, Delft, the Netherlands, personal communication, 2008).

An improved crumb softness was observed for most of the enzymes, compared to the control. The crumb softness of bread is influenced by the size of the bread slice, as less force will be needed

to compress a larger slice of bread than a denser, smaller slice. Therefore the most accurate means of determining softness is by baking lidded bread and testing the crumb softness (David Howard, Research and Development, Sasko, Paarl). The increased softness can therefore be attributed to the ability of some of the enzymes to increase the bread height and subsequently increasing the crumb softness. When the enzymes were combined during the small-scale baking trials, only an increase in bread height was observed.

Commercial scale (700 g) baking trials were conducted using a “no-time” straight dough bread making process. A lean formulation was used to study the effects of the enzymes. Some of the enzymes resulted in slightly sticky doughs. This could have been due to the high-speed mixer being used. The doughs, however, seemed to have recovered after a short resting period as they were all easy to handle during further processing. The pure endoxylanase and pure endoglucanase increased bread height significantly ( $P<0.05$ ). From the bread volume results, differences were observed between the manual measurements (rape seed displacement) and the C Cell measurements. The C Cell measurements are considered to be more accurate and reliable than the manual measurements as there is no human error involved. The C Cell results showed that only the pure endoxylanase and endoglucanase were able to increase bread volume significantly ( $P<0.05$ ). These results were similar to those observed for bread height, which confirms the more accurate measurements of the C Cell.

The pure endoxylanase resulted in a more open and coarser crumb texture. Both pure enzymes, however, resulted in a decrease in slice brightness. The combined commercial endoxylanase and endoglucanase resulted in a significant ( $P<0.05$ ) increase in bread height. The combined enzymes did not work synergistically in improving bread quality characteristics and individual enzymes could rather be used.

Both endoxylanases and endoglucanases can be used to improve bread quality characteristics such as bread height and/or volume, slice brightness and softness of crumb. Contradicting results were obtained to some extent between the small- and commercial scale baking trials. The formulations and processes used, however, differed and could have contributed to these results. In the small-scale trials it was noticed that pure endoxylanase improved bread height whereas pure endoglucanase improved slice brightness. The use of pure or single component enzymes could thus be beneficial as specific characteristics can be targeted. In addition, the bakeries could blend these single component enzymes to obtain desirable quality characteristics. A small number of single component enzymes are already commercially available. The use of these enzymes will become even more feasible if they can be supplied cost effectively.

Apart from testing the effect of the enzymes on bread quality characteristics using small-scale baking trials, testing of enzymes can also be conducted efficiently on commercial scale. The leaner formulation used, allowed for the effects of the enzymes to be observed more prominently. The benefit of the evaluation on commercial scale was that the effect of the enzymes was tested on a commercial flour and in a process similar to that used in industry.

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